

**The vulnerability of *ex vivo* α -motor nerve terminals to
hypoxia-reperfusion injury.**

By

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Declaration

I declare that the work described in this thesis and its composition are entirely my own unless otherwise stated, and the work has not been submitted for any other degree or professional qualification.

A handwritten signature in black ink, reading "R. Baxter". The signature is written in a cursive style with a large, stylized 'R' and 'B'.

Rebecca L. Baxter

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Abstract

A growing body of evidence shows that presynaptic nerve terminals throughout the nervous system are vulnerable to a range of traumatic, toxic and disease-related neurodegenerative stimuli. The aim of this study was to further characterise this vulnerability by examining the response of mouse α -motor nerve terminals at the neuromuscular junction (NMJ) to hypoxia-reperfusion injury. To address this aim, a novel model system was generated in which *ex vivo* skeletal muscle preparations could be maintained in an hypoxic environment, at an O₂ concentration below *in vivo* normoxic values (<0.25% O₂), for 2hr followed by 2hr reperfusion (2H-2R). Using this model system combined with quantitative assessment of immunohistological preparations as well as some ultrastructural observations, I present evidence to show that α -motor nerve terminals are rapidly and selectively vulnerable to hypoxia-reperfusion injury with no apparent perturbations to postsynaptic endplates or muscle fibres. I show that the severity of α -motor nerve terminal pathology is age and muscle type/location dependent: in 8-12wk old mice, nerve terminals in fast-twitch *lumbrical* muscles are more vulnerable than predominantly slow-twitch *transversus abdominis* and *triangularis sterni*. In 5-6 week old mice however, there is an age dependent increase in vulnerability of α -motor nerve terminals from the predominantly slow-twitch muscles while the fast-twitch lumbricals remained unaffected by age. The functional, morphological and ultrastructural pathology observed in α -motor nerve terminals following 2H-2R is indicative of selective and ongoing nerve terminal disassembly but, occurs via a mechanism distinct from Wallerian degeneration, as the neuroprotective *slow Wallerian degeneration* (*Wld^s*) gene did not protect nerve terminals from these pathological changes. I also provide provisional evidence to show that 1A/II muscle spindle afferents and γ -motor nerve terminals are more resistant to hypoxia-reperfusion injury compared with α -motor nerve terminals. In addition to this, I also report preliminary finding that indicate that the oxygen storing protein, neuroglobin, maybe expressed at the mouse NMJ and report the difficulties of using mice that express yellow fluorescent protein (YFP) in their neurons for repeat/live imaging studies. Overall, these data show that the model of hypoxia-reperfusion injury developed in this study is robust and repeatable, that it induces rapid, quantitative changes in α -motor nerve terminals and that it can be used to further examine the mechanisms regulating nerve terminal vulnerability in response to hypoxia-reperfusion injuries. These findings have clinical implications for the use of surgical tourniquets and in the aetiology of many neurodegenerative diseases and neuropathic sequelae where mechanisms relating to hypoxia and hypoxia-reperfusion injury have been implicated.

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Common Abbreviations

2H-2R:	2hr hypoxia followed by 2hr reperfusion
Ach:	acetylcholine
AChR(s):	acetylcholine receptor(s)
ADP:	adenosine diphosphate
ALS:	amyotrophic lateral sclerosis
AMP:	adenosine monophosphate
ATP:	adenosine triphosphate
BTX:	a-bungarotoxin
CHAT:	choline acetyl transferase
CNS:	central nervous system
EPP:	excitatory postsynaptic potential
FITC:	fluorescein isothiocyanate
GFP:	green fluorescent protein
LTX:	latrotoxin
MAP:	muscle action potential
MeOH:	methanol
MEPP:	miniature endplate potential
mSOD1:	mutant superoxide dismutase 1
NAD ⁺ :	nicotinamide adenine dinucleotide
NGB:	Neuroglobin
NF:	neurofilament 168kDa
NF/SV ₂ :	neurofilament 168kDa & synaptic vesicle 2 protein
NGF:	nerve growth factor
NMJ:	neuromuscular junction
nmnat1:	nicotinamide mononucleotide adenylyltransferase
PBS:	phosphate buffered saline
PNS:	peripheral nervous system
ROS:	reactive oxygen species
SDH:	succinate dehydrogenase
SV ₂ :	synaptic vesicle 2 protein
TA:	Transversus abdominis
TRITC:	tetramethylrhodamine isothiocyanate
TS:	triangularis sterni
Wld ^Δ :	Wallerian degeneration slow mutation
XFP:	colour-shift mutants of green fluorescent protein
YFP:	yellow fluorescence protein

Chapter 1

Introduction

1.1 General introduction

“...one common brief fundamental law ...: The nervous system consists of numerous nerve units (neurons), anatomically and genetically independent. Each nerve unit consists of three parts: the nerve cell, the nerve fiber and the fiber arborizations (terminal arborizations).”

(Waldeyer 1891)

The subject of neuroanatomy and pathology has a long history with documented evidence of its study extending as far back as the ancient Egyptians with the Edwin Smith Surgical Papyrus. Dating from c. 3000-1700 BC, this papyrus describes the symptoms of 48 patients many of whom had head wounds. The unknown Egyptian author annotated his clinical findings and suggested treatments (only one of which included magic) with observations of the brain, skull, cerebrospinal fluid and meninges (Feldman and Goodrich 1999). Our modern understanding of neuroanatomy and pathology however, has its foundations in the 19th century with the works of a number of eminent scientists, anatomists and clinicians, the most notable of which were Santiago Ramón y Cajal and Camillo Golgi (see Shepherd 1991) for a full historical review). During the 1880's Golgi discovered a technique that enabled neurons to be filled with silver chromate allowing for the first time the resolution required to explore the gross structural features of the nervous system that Golgi, Cajal and many others used to great effect. By the end of the 19th century, Wilhelm Waldeyer was able to combine the works and ideas of his peers with his skills of scientific interpretation and talent for terminology to produce the fundamental law of the nervous system translated above (Shepherd 1991). It is now unequivocally accepted that neurons are the highly specialized cells that form the basic unit of the nervous system and that they mediate the transfer and integration of electrical and chemical information from one part of an organism to another. It is however, worth noting that the neuron wasn't conclusively confirmed to be anatomically independent until the electron microscope was developed¹ and used in the 1950's to show that nerve terminals were truly separated from their target cells (Palade and Palay 1954; De Robertis and Danielli 1959). This finally gave the fine structural details to add to Charles Sherrington's theory of the 'synapse' in 1897 (Liddell 1952; Shepherd 1991). What many of these early scientists demonstrated is that clear and systematic study of neuronal structure gives important clues to neuronal function. The intrinsic relationship between structure and function can be demonstrated in pathological conditions where abnormal function i.e., the clinical manifestations, more often than not can be directly linked to some kind of structural change. As a result, observations of structural changes can provide an important starting point in determining and understanding pathological processes.

¹ The first prototype of the electron microscope was built by Ernst Ruska in 1931 and for his continual development of field of electron optics and microscopy; he was awarded the Nobel Prize for Physics in 1986. For their work on the structure of nervous system Golgi & Cajal shared a Nobel Prize in 1906 and for his work on the function of the nervous system Sherrington also received a Nobel Prize in 1932. (Nobelprize.org)

With our improved understanding of neurons, Waldeyer's concept of a neuron being composed of at least three structural parts or compartments appears to be increasingly important, particularly in regards to neuropathology. We now know that as well as being structurally different, the cell body, axon and nerve terminal may also be considered compartmentalised by their functional roles, metabolic properties and their vulnerability to pathological stimuli. Recent work in many traumatic, toxic and disease-related neurodegenerative conditions has highlighted the fact that nerve terminals appear to be highly vulnerable, with changes in their structure and function often appearing prior to changes in axons or cell bodies. The loss of nerve terminals undoubtedly causes a loss in neuronal connectivity and function, and therefore contributes to many of the punitive symptoms that characterise neuropathological conditions. One condition that affects up to 1 in 11,000 people in the UK is ischaemic paralysis following the routine use of surgical and non-surgical tourniquets (Kam 2007). This condition is characterised by long-term neurological and functional defects affecting α -motor neurons and their command of skeletal muscle, but the exact cause of this injury is still poorly understood. There is some indication that α -motor nerve terminals may be the principal site of tourniquet-induced paralysis and that hypoxia/ischaemia-reperfusion injury caused by tourniquets may be the principal stimulus. The potential involvement of α -motor nerve terminals in ischaemic paralysis and their vulnerability to hypoxia/ischaemia-reperfusion injury however, has yet to be conclusively addressed. This represents a serious gap in our knowledge and understanding of α -motor neurons, their nerve terminals and the cause of ischaemic paralysis following the use of tourniquets.

In the following chapter, I will review some of the evidence suggesting that α -motor nerve terminals are vulnerable to hypoxia/ischaemia-reperfusion injury. This includes their high energetic demands, the known vulnerability of nerve terminals, in general, to a range of pathological stimuli and the known vulnerability of nerve terminals to hypoxia/ischaemia-reperfusion injuries in the central nervous system (CNS). Finally, I will review our current knowledge of peripheral nerve hypoxia/ischaemia-reperfusion injuries and why it is still unclear if α -motor nerve terminals are vulnerable. I will however begin the discussion with a brief overview of why hypoxia (ischaemia) is a pathological stimulus and the basic structure and function of α -motor neurons and their nerve terminals. The rest of this study documents the progress made into resolving the issue of vulnerability of α -motor nerve terminals in response to hypoxia-reperfusion injury.

1.2 Oxygen & cellular respiration

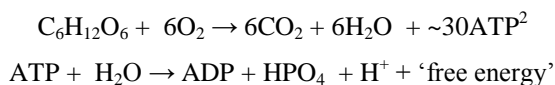
To determine if and how peripheral α -motor nerve terminals are vulnerable to hypoxia-reperfusion injury it is important to understand why hypoxia is a pathological stimulus. The following section therefore takes a very brief look at aerobic respiration and the role of oxygen to provide a basis for understanding the consequences of hypoxia-reperfusion injury at α -motor nerve terminals and to clearly introduce some important aspects of cellular metabolism that are referred to in later discussions. More detailed information can be found in a range of standard texts, including Alberts *et al.* (1989), Zubay (1998), Hanes and Hooper (2000), Lodish *et al.* (2008) which have been used in general reference in the following section.

1.2.1 Why is oxygen important?

Oxygen (O_2) is a colourless, odourless diatomic gas that makes up 21% of the atmosphere and is vital for aerobic respiration that sustains many forms of prokaryotic and eukaryotic life. Aerobic respiration is an exergonic process that liberates energy in the presence of O_2 from ingested/stored 'food'. This liberated energy is used to drive the phosphorylation of adenine nucleotides to generate the universal energy carrying molecule, adenosine triphosphate (ATP). The energy released by dephosphorylation of ATP into adenosine diphosphate (ADP) and to a lesser extent adenosine monophosphate (AMP) supplies all the cells endergonic processes, i.e., those processes that require energy, which maintain cellular homeostasis, morphology, and those that carry out generic as well as cell-specific functions.

1.2.2 Cellular respiration

The chemical fuel that supplies the energy for ATP synthesis can come from lipids, proteins or carbohydrates/sugars derived from ingested food. With limited cellular fuel stores, neurons almost solely rely on glucose supplied from the blood stream (Zubay 1998) and for this reason, only glucose metabolism will be considered here. The whole process of aerobic respiration using glucose as the fuel source and dephosphorylation of ATP can be summarized as:



The process of liberating the energy stored in glucose to ATP begins with the breakdown of glucose (a 6-carbon compound) to a pyruvate (a 3-carbon compound) via the process glycolysis which yields two molecules of ATP. Pyruvate is then further catabolised into carbon dioxide (CO_2 : a single carbon compound) in the Krebs' cycle (alternatively known as the citric acid cycle or the tricarboxylic acid cycle) that drives the phosphorylation of 1 molecule of ATP per turn of the cycle. Both glycolysis and

² There is considerable debate over the exact yield of ATP during aerobic respiration with values ranging from 29.5 to 36 molecules of ATP per glucose. I have elected for the conservative, whole number estimate of 30ATP's used by Zubay (1998).

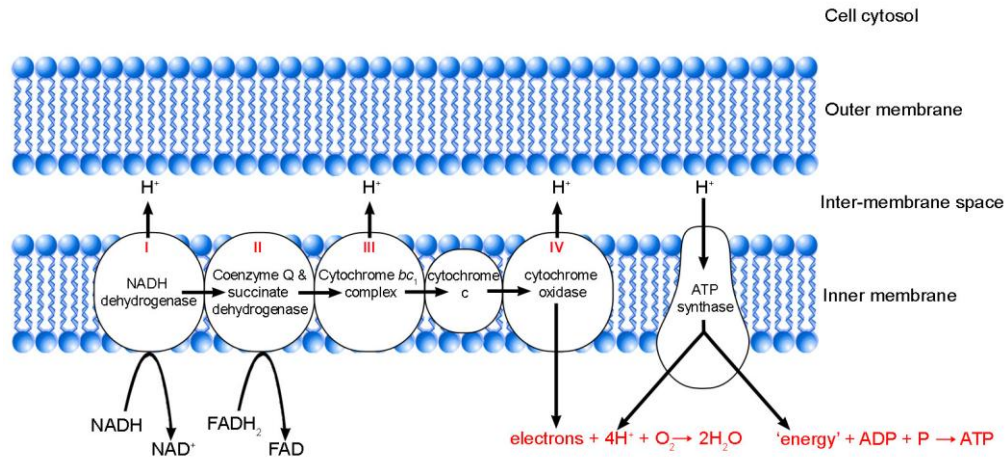


Fig 1.1 Overview of the electron transport chain and oxidative phosphorylation.

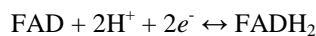
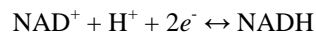
The four protein complexes (I, II, III and IV) and cytochrome c that constitute the electron transport chain are embedded in the inner-membrane of mitochondria. NADH and FADH₂ carry electrons from the sites of glycolysis and the Krebs' cycle to the electron transport chain where they are oxidised and release their electrons. As the electrons are passed down the chain they pump H⁺ into the inter-membrane space to form an electrochemical/concentration gradient. These H⁺ pass down their gradient via ATP synthase providing the energy required to drive the re-phosphorylation of ADP to ATP. The process is completed when the electrons are donated to O₂ in the presence of H⁺ to form H₂O.

the Krebs' cycle do not require oxygen i.e., they are anaerobic, and collectively yield four molecules of ATP. By harvesting electrons from glucose as it is catabolised to CO₂ and donating them to O₂, via the process of electron transport and oxidative phosphorylation that occurs in the mitochondria, a cell may increase this energy yield ~8 times.

1.2.3 Mitochondria, electron transport and oxidative phosphorylation

Mitochondria are large (1-10µm) bean-shaped, double-membrane bound organelles and their inner membrane is convoluted into a number of finger-like projection (cristae) that gives mitochondria their highly characteristic morphology. Mitochondria play an important role in buffering intracellular Ca²⁺ via a range of Ca²⁺ pumps and exchangers (e.g. Na⁺/Ca²⁺ exchanger) located on the mitochondrial membranes and can therefore modulate intracellular processes via Ca²⁺ signalling. Mitochondria can also mediate programmed cell death or 'apoptosis' by releasing cytochrome c (either by rupture or by the formation of pores) which activate caspases, a family of cysteine proteases that bring about the execution of cell death. The primary role of mitochondria however is electron transport and oxidative phosphorylation that supplies most of a cell's ATP. As glucose is broken down during glycolysis and the Krebs' cycle, spare electrons (e⁻) and H⁺ ions are harvested and bound to electron carriers. The electron carriers most commonly used by a cell during respiration are nicotinamide adenine

dinucleotide (NAD⁺) and flavin adenine dinucleotide (FAD), and as they gain electrons, they become reduced as follows:



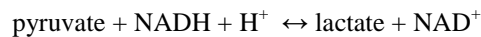
NADH generated by glycolysis in the cell cytosol and, NADH and FADH₂ generated by the Krebs' cycle in mitochondria transport e^- to the electron transport chain imbedded into the inner-membrane of mitochondria where they release their e^- and H^+ ions (i.e., they become oxidized) and return to the sites of glycolysis and the Krebs' cycle to harvest more e^- . The electron transport chain (see Fig1.1) is composed of four large protein complexes (I, II, III and IV) and cytochrome *c* that sequentially carry and exchange e^- to finally donate them to O₂ in the presence of H^+ to form H₂O. The energy generated by the exchange of e^- down the chain powers H^+ pumps and the movement of H^+ into the inter-membrane space to form a potential store of energy as an electrochemical/concentration gradient. As and when the cell requires, H^+ ions are allowed to flow down their H^+ electrochemical gradient, controlled by ATP synthase, to provide the energy to drive the phosphorylation of ADP to ATP. Collectively this process is known as oxidative phosphorylation and generates ~26 molecules of ATP per glucose, providing >86% of the total supply of ATP.

1.2.4 Why does hypoxia kill cells?

The average adult human will utilize ~60kg of ATP a day while a top marathon runner can expect to generate and consume a similar amount in a little over 2hr (Buono and Kolkhorst 2001). It would be almost impossible for humans or any organism to store the amount of ATP required to fuel daily activities and therefore they have to rely on the constant recycling of dephosphorylated ATP to fulfil their bioenergetic needs (Buono and Kolkhorst 2001). The most likely cause of insufficient ATP and a failure in cellular respiration is the inhibition of electron transport and oxidative phosphorylation due to the lack of oxygen. A lack of oxygen (hypoxia) can occur in a variety of different situations: environmental changes (e.g., at altitude), poor/intermittent gaseous exchange, or the result of reduced/impaired blood flow (ischaemia). The latter, ischaemia, is perhaps the best studied however, it is important to note that while many authors use the terms hypoxia and ischaemia interchangeably they represent related but distinct stimuli. Hypoxia refers to a lack of oxygen while ischaemia refers to a reduction/loss of the blood supply. Ischaemia therefore, not only encompasses hypoxia but also hypoglycaemia and other co-stimuli related to a loss or stagnation of blood supply. It is however the hypoxic element of ischaemia that is believed to be the major pathological stimulus.

In response to hypoxia/ischaemia cells are forced to reduce and adapt metabolic output as the supply of ATP becomes depleted. For a limited duration, cells may survive on already dissolved O₂ and ATP generated via anaerobic pathways, however insufficient oxidation of NADH and FADH₂ will also

eventually inhibit glycolysis and the Krebs' cycle. This can in part, be prevented by the conversion of pyruvate to lactate via lactate dehydrogenase.



The production of lactate and other adaptive responses can generally only buffer transient hypoxic/ischaemic insults as they cannot make up for the >86% of ATP generated via electron transport and oxidative phosphorylation. As ATP levels fall further, there will be an increasing failure of all ATP-dependent process and loss of cellular homeostasis. In particular, leak currents in the plasma membrane will go unchecked and accumulated ions (and other substances) will dissipate down their concentration gradients which can quickly lead to irrecoverable cellular damage and death (Hand and Menze 2008). Once cell death has become an inevitable outcome, residual ATP levels become pivotal in determining the mechanism of cell death. If the damage is severe and/or there is little or no available ATP, cell death will proceed by necrosis characterized by cellular swelling and lysis, mediated by Ca^{2+} proteolytic enzymes such as calpains. Less severe damage and/or residual levels ATP may allow for activation of an active, regulated program of cell death i.e., apoptosis characterized by cell shrinkage without lysis, typically mediated by mitochondrial release of cytochrome c and subsequent activation of caspases (e.g., Ankarcrona *et al.* 1995; Eguchi *et al.* 1997; Leist *et al.* 1997; Northington *et al.* 2007; Hand and Menze 2008; Mattson *et al.* 2008).

1.2.5 Summary

ATP is the major source of energy for most cells and the energy released by de-phosphorylation of ATP is used to fuel a range of activities from basic homeostasis to specific cell functions. The major source of ATP is from electron transport and oxidative phosphorylation in the mitochondria; however, this process relies heavily on a constant supply of O_2 . If O_2 becomes rate limiting (hypoxia) as commonly occurs due to a failure in the blood supply (ischaemia), ATP levels will quickly begin to fall causing a failure in all ATP-dependent process that can quickly lead to cell death. The remaining levels of ATP are then pivotal in determining the mechanism of cell death.

1.3. Structure and function of α -motor neurons

α -motor neurons have a specialized structure that allows them to carry out their specific role of controlling posture and commanding voluntary skeletal muscle movement (Fig1.2). In the following chapter, I review the basic structure of α -motor neurons to demonstrate that each compartment has specific structural characteristics related to their function. I will also briefly describe the functional and structural characteristics of skeletal muscle, as it is important to consider that efficient control of movement and posture relies on both muscle and α -motor neurons. Overall, this will provide the basis for understanding structural and functional response(s), especially of nerve terminals, to hypoxia-reperfusion injury. Further details, and used in general reference for the following section, can be found, for example in Kuffler *et al.* (1984), Kandel and Schwartz 1985, Alberts *et al.* 1989, Hall and Sanes (1993), Sanes and Lichtman (1999): Longstaff (2000) and Lodish *et al.* (2008).

1.3.1 Cell Body & Dendrites

The cell bodies of mammalian α -motor neurons are situated in the ventral horn of the spinal cord. The cell body is the location of the neuron's nucleus and synthetic machinery for the generation of proteins, enzymes, signalling molecules and many other macromolecules vital for cell survival and function. The dendrites of the cell body are the primary receptive region of α -motor neurons, receiving synaptic inputs from other neurons located within the CNS. The primary role of cell bodies is therefore to provide trophic support for the rest of the neuron and the coordination/initiation of electrical impulse to bring about muscle fibre contraction. In mammals, each motor neuron may command up to several thousand muscle fibres, where the motor neuron and the fibres that it innervates are collectively referred to as a 'motor unit' (Kandel and Schwartz 1985; Longstaff *et al.* 2000)

1.3.2 Axons

Inputs received onto the dendrites are conveyed by membrane depolarization (action potentials) via axons that track through the periphery to targeted skeletal muscle fibres. Axons typically bundle together to form 'nerves' that exit the spinal cord through the ventral roots. The primary role of axons is the propagation of action potentials from the cell body to the nerve terminal. One of the most salient features of motor neurons is their overall length and the axon, as a general rule in vertebrates, can extend approximately half the overall length of the animal (e.g., over a metre in humans or 10 metres in a whale: Bunge 1986). Their structure is therefore highly dependent on the cytoskeleton that provides mechanical/structural support for the maintenance of overall morphology and transportation of macromolecules and organelles to and from nerve terminals and cell bodies (reviewed by Wuerker and Kirkpatrick 1972). Neurofilaments, composed of polymerised light (~68kDa), medium (~168kDa) and heavy filaments (~200kDa), are the most stable of the three cytoskeletal components with a half-

life estimated to be 1-2 years and provide the most mechanical support (Petzold 2005). In direct contrast, microfilaments and microtubules are highly dynamic and undergo constant polymerisation and depolymerisation (treadmilling) of their constituent units, actin and tubulin respectively. Microfilaments and microtubules however can be stabilised with a variety of associated proteins and complexes so that they can provide the stability required for the transportation of macromolecules and organelles to and from nerve terminals and cell bodies (for reviews see e.g., Galbraith and Gallant 2000; Dent and Gertler 2003; Dillon and Goda 2005). To aid the speed and efficiency of action potential conduction along the length of the axon, Schwann cells wrap around and insulate axons in a myelin sheath and by leaving gaps of 1-2µm of exposed axon ('nodes of Ranvier'), action potentials progress down the axon in a saltatory manner. It is at these nodes therefore that high densities of the Na^+/K^+ ATPases, $\text{Na}^+/\text{Ca}^{2+}$ exchangers, voltage-gated Na^+ channels and others, that are responsible for polarisation and depolarization of the axon membrane are located.

1.3.3 The neuromuscular junction (NMJ)

First identified as early as 1830s by Valentin and Emmert, and famously studied by numerous eminent scientists including Kühne, Ranvier and Cajal (Lu and Lichtman 2007), the neuromuscular junction (NMJ) is the specialised communication point between α -motor neurons and muscle fibres. In the adult, each skeletal muscle fibre is innervated by only one collateral branch of an α -motor neuron. The NMJ is composed of the presynaptic α -motor nerve terminal, the postsynaptic endplate³ located on the muscle fibre and terminal Schwann cells. For this reason, it is often referred to as being a 'tripartite' synapse and is found in a depression in the central region of each muscle fibre. The primary role of the NMJ is the conversion of the electrical impulses/action potentials initiated at the cell body and conveyed by the axon into a chemical signal that can trigger the contraction of muscle fibres.

α -motor nerve terminals

α -motor nerve terminals are formed by a series of small closely packed varicosities or 'boutons', approximately 1-5µm in diameter linked by fine unmyelinated axons. Collectively, these boutons form the arborisation of the nerve terminal that in adult rodents typically ranges from ~20 to 50µm in size. Nerve terminals are loaded with up to ~170,000 synaptic vesicles (Reid *et al.* 1999), ~40nm diameter organelles, that cluster towards and bind with synaptic contact points or active zones on regions of the presynaptic bouton membrane that directly oppose post-synaptic specialisations. Upon the arrival of an action potential and the influx of Ca^{2+} via voltage gated Ca^{2+} ion channels, vesicles bound to presynaptic active zones and that have been primed to respond to Ca^{2+} undergo fusion-pore opening with the presynaptic membrane to release chemical neurotransmitters into the synaptic cleft.

³ In some of the early studies of NMJ morphology, Wilhelm Krause (1863) coined the term "motorische endplatte" (muscle endplate) referring to the entire NMJ, both pre- and postsynaptic (Lu and Lichtman 2007). While the term endplate is still in use, in more recent times it has been increasingly used to refer to just the specialised postsynaptic structures on the muscle fibre. For this reason, in this study the term endplate is also only used to identify the postsynaptic specialisations.

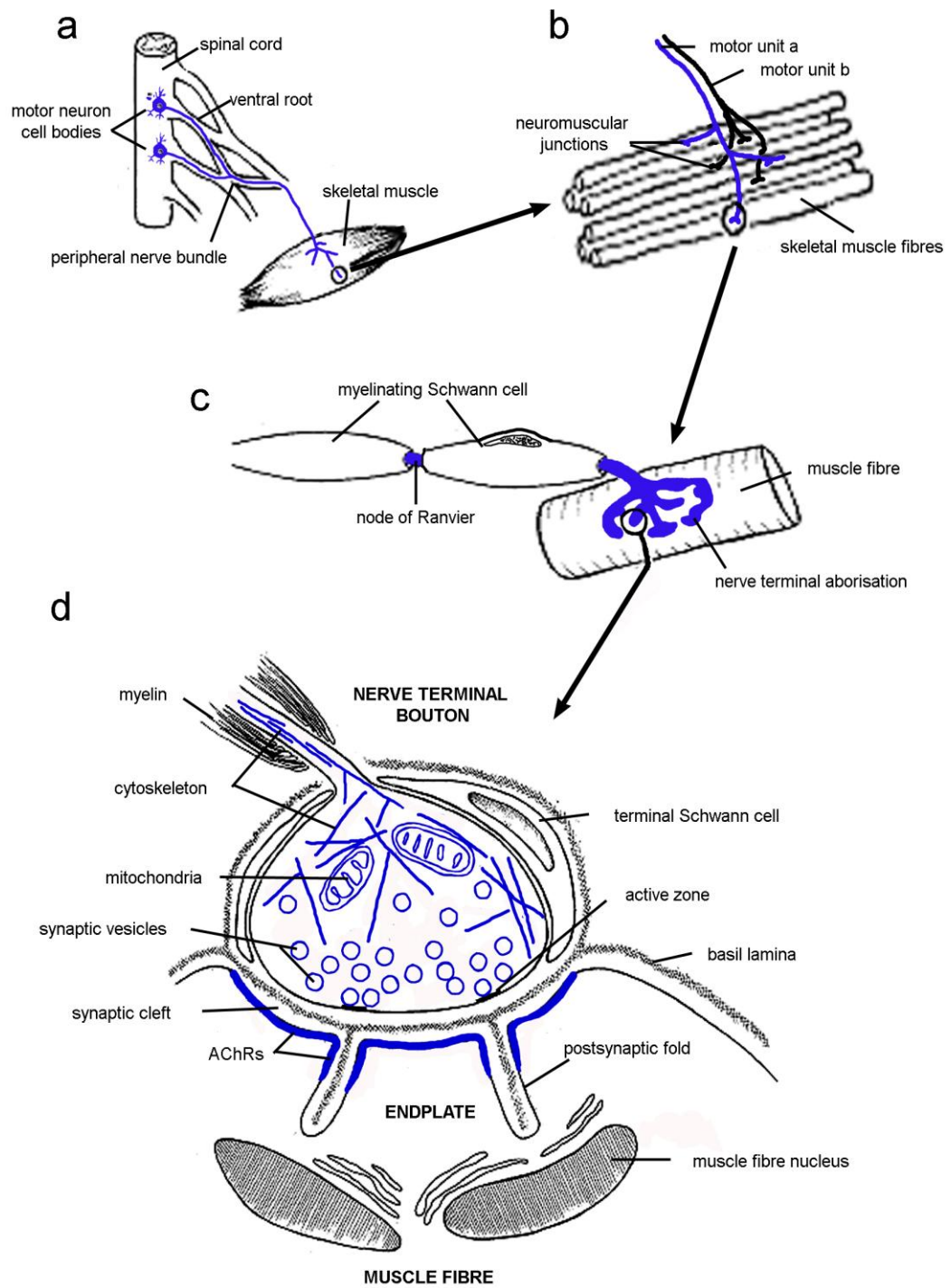


Fig 1.2 Organisation of α -motor neurons and the neuromuscular junction. a)

The cell bodies of α -motor neurons are located in the spinal cord and project axons in the periphery to innervate skeletal muscle. **b)** Neuromuscular junctions are the specialised communication point between α -motor neuron and skeletal muscle. In the adult, each muscle fibre is innervated by one α -motor neuron but each α -motor neuron innervates several muscle fibres to form motor units. **c)** The nerve terminal of α -motor neurons forms an arborisation on each muscle fibre. **b)** The nerve terminal arborisation is composed of series of boutons that synapse with skeletal muscle fibres. The presynaptic membrane of the nerve terminal boutons contains active zones that are the sites of synaptic vesicle release. Separated by the synaptic cleft and opposing the presynaptic membrane is the postsynaptic endplate of the muscle fibre that is highly folded with high densities of acetylcholine receptors (AChRs). Figure adapted from Hall and Sanes 1993.

his process of exocytosis is then followed by endocytosis, whereby empty vesicles are recycled and refilled with neurotransmitter chiefly acetylcholine (ACh) but also compounds including ATP. The ACh released by nerve terminals traverses the ~50nm synaptic cleft that contains the synaptic basal lamina and separates the pre- and postsynaptic apparatus and binds to postsynaptic nicotinic acetylcholine receptors (AChRs).

To limit the duration of action, ACh is deactivated by acetylcholinesterase bound to the synaptic basal lamina into acetate and choline, the latter of which is transported back into the nerve terminal. Further metabolism by several enzymes, including choline acetyltransferase (CHAT: first identified by (Nachmansohn and Machado 1943), allows regeneration of acetylcholine stores to refill synaptic vesicles.

Postsynaptic endplates

Directly opposing the α motor nerve terminal is the postsynaptic specialisation or muscle fibre endplate. The endplate is composed of a highly folded membrane with AChRs densely packed on the top of crests directly opposing the presynaptic active zones and voltage gated Na^+ channels located at the base of the folds. Activation of AChRs by acetylcholine allows influx of Na^+ and K^+ to trigger a localised depolarisation known as an endplate potential (EPP), that if above threshold activates voltage gated Na^+ channels to depolarise the whole muscle fibre generating the muscle action potential (MAP). Spontaneous release of single vesicles from active zones trigger miniature endplate potentials (MEPP) that are not above threshold and do not trigger whole muscle fibre depolarisation.

Schwann cells

Once the axon starts to specialize into the nerve terminal, myelinating Schwann cells are replaced by up to four unmyelinating terminal Schwann cells that completely overlie the nerve terminal but do not intrude into the synaptic cleft (Palade and Palay 1954; Desaki and Uehara 1981). Schwann cells are electrically non-excitable so do not play a direct role in neurotransmission but they have an important role in the development, maintenance, plasticity and pathological responses of motor neurons and their nerve terminals.

1.3.4 Muscle Fibres (and Muscle Spindles)

Skeletal muscle is formed from the fusion of multiple myoblasts into multinucleated, elongated fibres that are ~10-100 μm in diameter and can extend the length of the muscle of which they form a part. A thin layer of connective tissue encases each muscle fibre (endomysium) and groups of muscle fibres (perimysium) to form a continuous network of connections with the thick outer layer of muscle (epimysium) and tendons to which muscles attach to the skeleton. Viewed under a light microscope, skeletal muscle fibres have a striated appearance due to the highly ordered array of cytoskeletal filaments that are responsible for their contractile properties. These filaments bundle together to form myofibrils and are composed of actin and polymerised myosin molecular motors or thin and thick

filaments respectively (Fig1.3&4). In response to muscle fibre depolarisation and release of Ca^{2+} from sarcoplasmic reticulum, the myosin heads of thick filaments bind to and slide actin filaments to shorten the muscle fibre and cause contraction. Also contained within most muscles are a group or groups of weak 'intrafusal' muscle fibres that do not contribute to the force of contraction but provide sensory feedback concerning changes in the force and velocity of the muscle contraction/relaxation via specialised sensory neurons. These fibres are encased in a thick, fluid filled "capsule" composed of alternating layers of collagen fibrils (which either encircle or run longitudinally) and layers of specialised "capsular sheet cells" that is believed to form a mechanical and metabolically-active barrier to isolate and protect the specialised sensory endings located on the intrafusal muscle fibres (Barker 1974). IA/II nerve terminals located on the non-contractile equatorial regions of the intrafusal muscle fibres fire action potentials in response to the degree of muscle fibre stretch (i.e., they are mechanoreceptors rather than synapses). Located towards the contractile polar regions of the intrafusal fibres are small γ -neuromuscular junctions that help to maintain sensory feedback by triggering intrafusal muscle fibre contraction to keep the equatorial regions of the fibres taut so that it can continue to respond to muscle stretch (Barker 1974; Longstaff 2000: Fig 1.4). Collectively these intrafusal muscle fibres and IA/II and γ innervations are called the muscle spindle whose primary role is the coordination of antagonistic muscle contraction and proprioception, especially important in stabilising posture, coordinating gait and scaling responses to stimuli (e.g., van Deursen and Simoneau 1999; Longstaff 2000; Muller *et al.* 2008).

1.3.5 Summary

α -motor neurons, as well as all other neurons, can be thought of as being anatomically and functional compartmentalised. Cell bodies and dendrites located in the spinal cord are responsible for the initiation of action potentials that are propagated through the periphery by axons to elicit skeletal muscle contraction by chemical transduction at α -motor nerve terminals located on the centre of muscle fibres. These anatomical and functional characteristics of α -motor neurons, alongside those of skeletal muscle and the muscle spindle, collectively coordinate efficient control of voluntary movement and the maintenance of posture. As a result of these different anatomical and functional compartments of neurons, there may also be compartmentalisation of other properties such as bioenergetic properties and vulnerability to pathological stimuli.

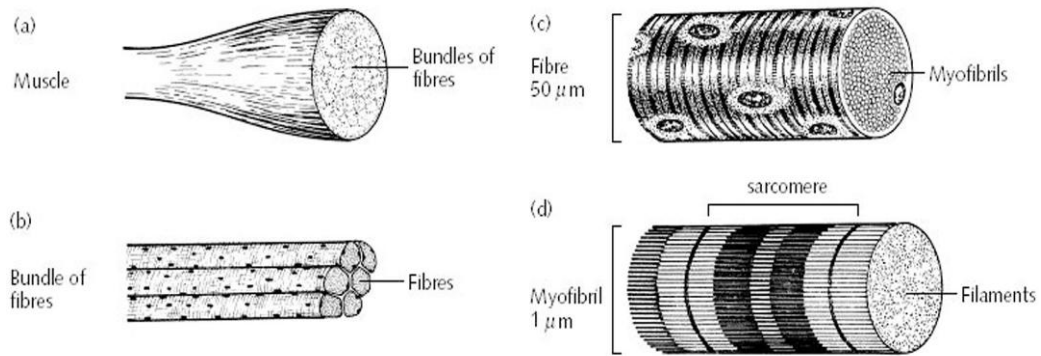
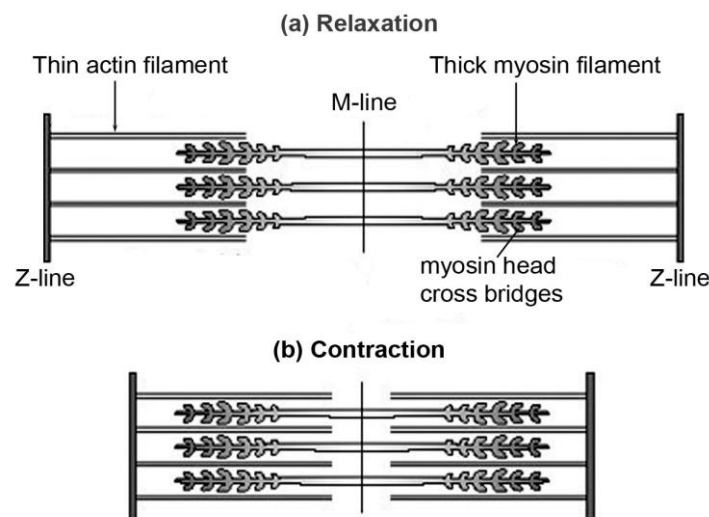


Fig 1.3 (above): Overview of skeletal muscle structure at increasing magnification. (a) the whole muscle; (b) bundle of muscle fibres; (c) a single muscle fibre, composed of myofibrils, showing nuclei and striations; (d) a single myofibril, composed of myofilaments. The regular arrangements of filaments across the whole muscle fibre accounts for the striated appearance of skeletal muscle when examined with a microscope and are responsible for bringing about contraction. Figure adapted from answers.com that was originally adapted from Jennett, S. (1989). Human physiology. Churchill Livingstone, Edinburgh.

Fig 1.4 (below): Overview of filament arrangement in skeletal muscle. (a) when relaxed and (b) when shortened during contraction. Myofibrils are composed of a repeating unit or sarcomere shown below (also identified in Fig 1.3(d) above) composed of thin actin filaments and thick myosin filaments that appear as alternating light and dark bands. A sarcomere is defined as extending from one z-line to another which can be identified as a thin dark band (see Fig 1.3(d) above) and the centre of a sarcomere is identified by another thin dark band called the m-line. During muscle contraction, the myosin heads on the thick filaments form cross bridges with the actin filaments to cause the filaments to overlap to a greater degree, decreasing the distance between z-lines. As this occurs in all sarcomeres the whole skeletal muscle shortens or contracts. Figure and legend adapted www.answers.com/topic/skeletal-muscle.



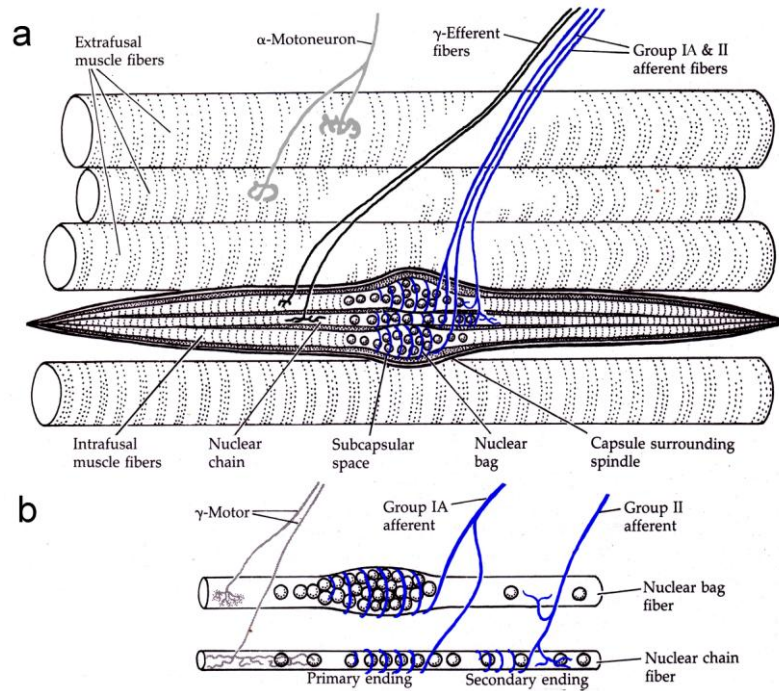


Fig 1.5: Overview of the mammalian muscle spindle. (a) Intrafusal fibres and spindles are encased in a capsule which is embedded in the muscle and surrounded by force generating extrafusal fibres. (b) There are two types of intrafusal fibres according to the arrangement of muscle fibre nuclei: bag and chain. IA afferent neurons form annulospiral endings surround the equatorial regions of the intrafusal fibres (primary endings). II afferent neurons can also form annulospiral endings in sub-equatorial regions of the chain fibres (secondary endings). γ -motor efferent neurons form either plate or trails of small γ -neuromuscular junctions at polar ends of the intrafusal fibres. These γ -neuromuscular junctions help ensure that the equatorial regions of the intrafusal muscle fibres remain taut and able to respond to the degree of stretch (Figure with minor adaptations Kuffler *et al.* 1984).

1.4 Bioenergetics & Metabolism: implications for α -motor neurons

Neurons and skeletal muscle have some of the highest workloads and metabolic rates of all tissues. For example, skeletal muscle may increase its demand for ATP >100 times per unit of time during exercise compared to when at rest (Kernell 2006), while the brain consumes approximately 20% of all inspired oxygen (reviewed by Rolfe and Brown 1997). Based on the previous chapter where I described the basic structure and function of α -motor neurons and skeletal muscle, the following chapter discusses how different functional attributes appear to lead to metabolic compartmentalisation within neurons. I will go on to discuss how metabolic characteristics differ between different skeletal muscle fibre types and review some of the evidence that suggest a reciprocal relationship between α -motor neurons and skeletal muscle fibres.

1.4.1 Bioenergetic and metabolic properties of neurons

Neurons gain nearly all of their energy from the oxidative phosphorylation of ATP in mitochondria (Ames 2000) and depolarization induced increases in O_2 consumption, glucose utilization and CO_2 production have been known for decades (Gerard 1930; Bak *et al.* 2006). It has been estimated that only 25-35% of a neuron's total energy production is used to fuel generic house-keeping tasks while the remaining 65-75% of a neuron's energy expenditure is used for signalling processes (Attwell and Laughlin 2001; Du *et al.* 2008). Little is known about the energy demands of specific process but at nerve terminals for example, conversion of choline and acetyl-CoA to acetylcholine via CHAT is energy dependent (Nachmansohn and Machado 1943), filling of synaptic vesicles with neurotransmitter is dependent on H^+ -ATPase pumps (e.g., Stadler and Tsukita 1984; Haigh *et al.* 1994) and two vesicular proteins important for endocytosis, Synaptic Vesicle 2 (SV_2) protein and synapsin have been shown to have ATPase activity/ATP binding sites (Hosaka and Sudhof 1998; Yao and Bajjalieh 2008). The metabolic cost of vesicle docking, fusion pore opening and recycling has been calculated to require at least 400 molecules of ATP per released vesicle (Attwell and Laughlin 2001). Using all the available information concerning the energy dependent process in the predominantly glutamatergic grey matter of the rodent brain, Attwell and Laughlin (2001) identify that neurotransmitter recycling, vesicle recycling and clearance of Ca^{2+} from nerve terminals accounts for ~7% of the total energy expenditure. The highest energy consuming process, accounting for the >90% remainder of Attwell and Laughlin signalling energy budget, is maintaining and re-establishing polarised membranes, with >40% of total oxygen consumption thought to be coupled to the Na^+/K^+ ATPase exchanger alone (Astrup *et al.* 1981; Erecinska and Silver 1989). While only 7% of the total energy used in neuronal signalling is utilized at the nerve terminal, when this is added to the regional cost of maintaining and re-establishing membrane potentials, the result is a high localized demand for ATP. Cytochrome oxidase activity and mitochondrial density at nerve terminals is second only to that found in the dendritic trees reflecting the high energy demands of these compartments, while axons, with the lowest overall energy requirements contain the lowest levels of mitochondria and cytochrome oxidase activity (Wong-Riley 1989; Hollenbeck and Saxton 2005). Further

compartmentalization of metabolism is reflected in the distribution of neuroglobin, a recently identified neuron-specific oxygen storing molecule related to myoglobin and haemoglobin (Burmester *et al.* 2000; Dewilde *et al.* 2001) which has also been shown to localize in cell bodies and dendrites with little present in axons, but unfortunately nerve terminals were not studied (Hundahl *et al.* 2008); see Chapter 5). It has also recently been shown that mitochondria with the highest potentials that drive the oxidative phosphorylation of ATP are transported towards nerve terminals while those with the lowest potentials are transported towards cell bodies where they are probably degraded (Miller and Sheetz 2004). This has added to the general opinion that there are also fundamental differences in synaptic mitochondria versus non-synaptic mitochondria and that these differences in turn differ between neurons from different brain regions e.g., cortex and hippocampus (e.g., Blokhuis and Veldstra 1970; Lai *et al.* 1977; Dagani *et al.* 1983; Leong *et al.* 1984; Dagani *et al.* 1988; Kiebish *et al.* 2008). More recent studies investigating the threshold activities of the mitochondrial complexes (see section 1.2.3 and Fig 1.1) have shown that to cause an abrupt decrease in oxidative phosphorylation and depletion of ATP, complexes I, III, and IV in non-synaptic mitochondrial and III and IV in synaptic mitochondria need to be inhibited by ~60-80%. Complex I in synaptic mitochondria however has a significantly lower threshold for inhibition at 25%, indicating that subtle changes in metabolism will cause a significant decrease in ATP production (John 1996; Davey *et al.* 1998). Synaptic mitochondria have also been shown to have reduced Ca^{2+} buffering capacity, which is probably related to the higher cyclophilin D content (Brown *et al.* 2006; Naga *et al.* 2007). This reduction in Ca^{2+} buffering capacity is suggested to be a consequence of accumulated oxidative damage and mitochondrial ageing. This may occur as they are transported to terminals and makes the formation of large mitochondrial pores more probable resulting in swelling and mitochondrial rupture (Brown *et al.* 2006). Overall this evidence indicates that neurons conserve energy by determining oxidative capacity by functional attributes at the cellular level as well as at the local level (Wong-Riley 1989) which is consistent with the idea that to improve efficiency and overcome poor cytoplasmic diffusion, sites of ATP generation must oppose sites of ATP consumption (Ames 2000; Hollenbeck and Saxton 2005). Unfortunately there are no comparable studies in α -motor neurons in the peripheral system, however it is known that α -motor nerve terminals also have a high density of mitochondria with more complex cristae compared to axons (Palade and Palay 1954; Vial 1958) and in the frog, mitochondria can occupy up to 38% of terminal bouton volume (Alnaes and Rahamimoff 1975).

1.4.2 Reciprocal properties of α -motor neurons and muscle fibres

As well as having different functional attributes determining metabolic demands at the local and cellular levels some of a neuron's function, structure and therefore metabolic properties may also be determined by their post-synaptic targets. In the case of α -motor neurons these post-synaptic targets are skeletal muscle fibres, which are well known to vary their properties to match functional demands.

Some skeletal muscles are tonically activated requiring long-term contraction, such as postural muscle, while others are used for making more rapid, short term movements. Those muscle fibres belonging to the latter group are predominantly composed of fast-twitch muscle fibres (also referred to as type II), which contract and relax more rapidly compared to tonically activated slow-twitch muscles fibres (also referred to as type I). The activation patterns of these two groups of muscle fibres are also reflected in their metabolic properties and fatigability. Slow-twitch muscle fibres rely predominantly on aerobic (oxidative) respiration, have more mitochondria and associated proteins/complexes, more myoglobin, larger capillary networks and are relatively fatigue resistant, which suits long term activation. Conversely, fast-twitch muscle fibres rely predominantly on anaerobic respiration and therefore have more enzymes relating to anaerobic pathways, fewer mitochondria, less myoglobin, smaller capillary networks but will quickly fatigue in response to activity and are therefore, more suited to quick bursts of activity (e.g., Ranvier 1874; Ogata and Mori 1964; Close 1972; Ogata and Yamasaki 1985; Rolfe and Brown 1997; Kernell 2006). It is important to note however that classification of muscle fibre type is highly complex with differing criteria and classification systems depending on molecular myosin isoforms, ATPases and a range of physiological parameters. Furthermore, fibre types are not easily separated into clear groups as they appear to exist on a continuum between fast and slow extremes (Kernell 2006) and it is for this reason and the purpose of this study muscles and their fibre types will be simply classified as either 'fast' or 'slow'.

The differences in fibre type properties between fast and slow muscles appear also to be reflected in some of the anatomical and functional properties of innervating α -motor neurons. In comparison to α -motor neurons that innervate fast-twitch muscle, there is a tendency for α -motor neurons innervating slow-twitch muscle fibre types to have smaller cell bodies and axons with corresponding slower conduction velocities than those innervating fast-twitch muscles that are used for rapid and more powerful motor activities (Campa and Engel 1971; Kernell 2006). Furthermore, α -motor neurons innervating slow-twitch muscles also have reduced repetitive firing rate but also require smaller excitatory currents to induce muscle fibre contraction which is advantageous for continuing tonic activation of slow-twitch, postural muscles (Hennig and Lomo 1985; Wood and Slater 1995; 1997; Kernell 2006). There also appears to be a difference in NMJ morphology according to fibre type with NMJs on fast-twitch fibres being smaller but having larger numbers of AChRs and releasing more synaptic vesicles that enhances efficiency and contributes to the larger safety factor of neurotransmission (Sterz *et al.* 1983; Fahim *et al.* 1984; Wood and Slater 1997; 2001; Bewick *et al.* 2004). Conversely, α -motor nerve terminals from slow-twitch muscles have larger pools of synaptic vesicles facilitating long term neurotransmission required to maintain tonic/repetitive muscle contraction (Reid *et al.* 1999; Bewick *et al.* 2004). This apparent 'reciprocal' relationship between fibre type and motor neurons, or covariance of functional and anatomical properties (Kernell 2006) has led to several studies to try and determine if neuronal oxidative capacity correlates with muscle fibre type. Comparison of succinate dehydrogenase (SDH, a key enzyme in the mitochondrial

respiratory chain see fig1.1) in α -motor neuron cell bodies shows that there are differences in metabolic capacity across motor neurons and between motor neurons that innervate the same muscle. Motor neurons that have larger cell bodies and innervate fast-twitch anaerobic fibres are consistently found to have low levels of SDH, while those with small cell bodies innervating slow-twitch oxidative muscles tend to have higher SDH levels (Campa and Engel 1971; Donselaar *et al.* 1986; Ishihara *et al.* 1988; Chalmers and Edgerton 1989; Chalmers *et al.* 1992; Ishihara *et al.* 1995; Nakano *et al.* 1997, (c.f. Roy *et al.* 2007)). This would suggest that larger motor neurons that innervate fast-twitch muscles rely less on energy supplied via mitochondria.

1.4.3 Summary

Neurons in general have high energy demands but there is evidence to suggest that there is distinct regionalization of bioenergetic needs, with nerve terminals requiring a large amount of ATP to supply their functional demands. This high bioenergetic need of nerve terminals is also combined with evidence that would indicate that the mitochondria located in nerve terminals are more susceptible to inhibition and pathological stimuli. Additionally, due to increased length of time in transit, increasing age and accumulation of oxidative damage, the mitochondria in nerve terminals of the longest neurons, such as α -motor neurons, may be more susceptible. Overall, this would indicate that the nerve terminal compartment of neurons and in particular, those of α -motor neurons would be highly vulnerable to metabolic inhibition such as that occurring during an hypoxic/ischaemic insult. Further to this, it may be predicted that there is a difference in α -motor nerve terminal vulnerability according to muscle fibre type. Different skeletal muscles fibre types and α -motor neurons are recruited in a task-dependent manner and therefore share patterns of activity. Based on this and evidence that indicates α -motor neurons co-vary many of their anatomical and functional parameter to match that of their innervated muscle fibre type, there may also be co-variance of metabolic parameters. Slow-twitch muscle fibres rely predominantly on aerobic respiration while fast-twitch muscle relies on more anaerobic respiratory pathways. This would suggest that according to muscle fibre subtype and therefore perhaps their innervating motor neurons, there may be significantly different responses to hypoxic/ischaemic insults.

1.5 Pathological responses of α -motor neurons

As a conservative estimate, but excluding psychiatric disorders such as depression, 6% of the UK population suffers from a neurological disorder at least once in their lifetime (MacDonald *et al.* 2000). The morbidity and socio-economic burden of these neurological conditions, such as stroke, diabetic neuropathies, compressive neuropathies, congenital diseases and major injury, which all appear within the top 10 of the most common conditions in the UK (MacDonald *et al.* 2000), has driven a significant amount of research in their neuropathogenesis and the mechanisms of neuronal death. This has revealed that there appears to be specific cellular mechanisms relating to the loss of cell bodies and dendrites, axons, and in particular, nerve terminals. In the following section, I will discuss some of the evidence that highlights the compartmentalisation of pathological response in α -motor neurons with a particular focus on α -motor nerve terminals to demonstrate that nerve terminals are a highly vulnerable compartment.

1.5.1 Wallerian degeneration

Structural characteristics

Due to their length, the peripheral axons of α -motor neurons are highly susceptible to mechanical trauma such as transection (axotomy) or crush injuries. This coupled with their experimental accessibility means that the pathological response of α -motor neurons to such injuries is historically well established. One of the first to study the effects of axotomy was Augustus Waller who, in 1850, published a study describing the loss of sensory reflexes and motor control of the frog tongue after glossopharyngeal and hypoglossal axotomy (Waller 1850). Waller documented the progressive fragmentation and eventual removal of debris from the nerves distal to the site of injury (distal nerve stump), a process now known to occur in a range of species including humans (Chaudhry and Cornblath 1992) and referred to as 'Wallerian degeneration'. The principal event of Wallerian degeneration is functional failure due to loss of connectivity but the distal nerve stump can continue to conduct axon potentials and release synaptic vesicles in rodents for up to 6-10hr *in vivo* or 20hr *ex vivo* at 25°C following axotomy (Miledi and Slater 1968; 1970; Winlow and Usherwood 1976; Tsao *et al.* 1994; Tsao *et al.* 1999; Gillingwater and Ribchester 2001). Following this lag phase, there is a loss of action potential propagation at 12-24hr *in vivo* (Miledi and Slater 1968; 1970) or >24hr at 25°C *ex vivo* (Tsao *et al.* 1999) and degenerative events are observed at the α -motor nerve terminal. At rodent α -motor nerve terminals this includes progressive disruption and lysis of mitochondria, activation of Schwann cells with processes encroaching into the synaptic cleft, aggregation and loss of synaptic vesicles, loss of neurofilaments, vacuoles and disruption of the presynaptic membrane (Birks *et al.* 1960; Miledi and Slater 1968; 1970; Winlow and Usherwood 1975; 1976; Gillingwater and Ribchester 2001). Signs of axonal degeneration swiftly follow, with microtubule depolymerisation and neurofilament degradation respectively preceded by the retraction of the myelin sheath from the

nodes of Ranvier and the cytoplasm becoming electron dense. Both axons and nerve terminals become fragmented into small ‘ellipsoid’ membrane-bound chambers that undergo myelomonocytic invasion and engulfment by Schwann cells to be cleared away over a period of a few weeks (Vial 1958; Birks *et al.* 1960; Schlaepfer and Hasler 1979; Glass and Griffin 1991; Gillingwater and Ribchester 2001; Zhai *et al.* 2003; Beirowski *et al.* 2005). These degenerative events in axons and nerve terminals tend to be matched with apoptotic death of cell bodies in young/neonatal animals up to ~2wks of age (e.g., Adalbert *et al.* 2006). In adults however, there is some retraction of the dendrites that may be permanent (Sumner and Watson 1971; Brännström *et al.* 1992) but the cell body normally survives and may eventually re-innervate its skeletal muscle target (Waller 1852; Kashihara *et al.* 1987; Johnson and Duberley 1998; Gillingwater and Ribchester 2001; Tiraihi and Rezaie 2004). Postsynaptic endplates, AChRs and muscle fibres are largely unaffected by Wallerian degeneration in the short term, where for at least 3 weeks following axotomy no structural changes in the endplate are apparent but depolarisation of muscle fibres can be observed 3hr after axotomy (Birks *et al.* 1960; Miledi and Slater 1968; Bray *et al.* 1976). Following a few days to weeks there is increasing muscle fibre atrophy (Miledi and Slater 1968; Schmalbruch *et al.* 1991a), peri-junctional AChRs increase and there is increased turnover of AChRs at the endplate (Szabo *et al.* 2003; Kernell 2006).

Mechanism of Wallerian degeneration: The *Wld^s* mutation

The mechanism(s) underlying Wallerian degeneration are still unclear. Despite its highly ordered execution, Wallerian degeneration does not appear to be linked with apoptosis as studies have ruled out caspases-3 activation, the primary apoptotic mediator in neurons (Finn *et al.* 2000) or involvement of other pro-apoptotic factors such as Bcl-2 (Sagot *et al.* 1995) Bax and Bak (Whitmore *et al.* 2003). Wallerian degeneration can however be inhibited in cultured dorsal root ganglia by decreasing Ca^{2+} concentration and with calpain inhibitors (Glass *et al.* 1994; George *et al.* 1995; Wang *et al.* 2000). The serendipitous discovery by Lunn *et al.* (1989) that Wallerian degeneration can be inhibited in the CNS and PNS for up to 3 weeks and that impulse conduction and transmitter release can be maintained for >3 days by the autosomal dominant mutation known as the *Wallerian degeneration slow* gene (*Wld^s*), has forced some significant conceptual changes regarding Wallerian degeneration and mechanisms of neuronal death (Lunn *et al.* 1989; Perry *et al.* 1990; Tsao *et al.* 1994; Ribchester *et al.* 1995; Adalbert *et al.* 2005). This tandem triplication mutation causes an additional open-reading-frame on mouse chromosome 4 and transcribes a novel 85kd in-frame fusion protein consisting of nicotinamide mononucleotide adenylyltransferase (Nmnat1: a NAD^+ synthesising enzyme), a unique ‘*Wld^s* -18’ linking sequence, and the last 70 N-terminal amino acids of Ube4b, a ubiquitination enzyme responsible for regulated protein degradation (Lyon *et al.* 1993; Coleman *et al.* 1998; Conforti *et al.* 2000; Mack *et al.* 2001; Gillingwater *et al.* 2002; Adalbert *et al.* 2005). How this mutation confers protection against Wallerian degeneration is still unknown, especially as the transcribed *Wld^s* protein is thought to remain exclusively in the cell body (Gillingwater and Ribchester 2001; Mack *et al.* 2001; Fang *et al.* 2005, although this has recently been disputed with evidence of a cytoplasmic/mitochondrial distribution of the *Wld^s* protein by Beirowski *et al.* 2009). It does appear

that both Nmnat1 and Ube4b are required to prevent axonal degeneration (Coleman 2005; Conforti *et al.* 2007b; Watanabe *et al.* 2007 (c.f. Sasaki *et al.* 2009a)) and synergy between the proteins ability to modulate NAD⁺ synthesis and the ubiquitin-proteasome system have been suggested as possible mechanisms of inhibition (Mack *et al.* 2001; Raffaelli *et al.* 2002; Zhai *et al.* 2003; Araki *et al.* 2004; Coleman and Ribchester 2004; Adalbert *et al.* 2005; Wang *et al.* 2005; Laser *et al.* 2006; Sasaki *et al.* 2006; Conforti *et al.* 2007b). Alternatively, more recent work indicates that the *Wld^s* protein may alter cell cycle status (Wishart *et al.* 2008) and gene transcription relating to cell stress responses (Gillingwater *et al.* 2006b; Laser *et al.* 2006; Wishart *et al.* 2007), axonal transport proteins (Simonin *et al.* 2007) as well as transcription/distribution of other gene products (Gillingwater *et al.* 2006b; Laser *et al.* 2006). What the *Wld^s* mutation shows however is that Wallerian degeneration is unlikely to be a passive process but rather a genetically regulated intrinsic program of axonal and nerve terminal loss that, at the molecular level, is distinctive from apoptosis (Gillingwater and Ribchester 2001; Raff *et al.* 2002; Coleman 2005; Low and Cheng 2005; Luo and O'Leary 2005; Wishart *et al.* 2006; Saxena and Caroni 2007). This is most clearly suggested by studies that show that the rate of Wallerian degeneration can be modulated by a transgene in a 'dose' dependent manner (Mack *et al.* 2001) and that *Wld^s* mediated neuroprotection can be completely lost in 'wasted' mice lacking a key protein synthesising/elongation factor (eEF1A2: Murray *et al.* 2008b), that shows Wallerian degeneration relies on transcriptional processes.

Wallerian degeneration in pathological processes

As well as providing protection against mechanical trauma the *Wld^s* mutation has been shown to inhibit degeneration caused by: disruption of microtubules (Luduena *et al.* 1986; Wang *et al.* 2000; Wang *et al.* 2001), progressive motor neuropathy (Ferri *et al.* 2003; Simonin *et al.* 2007), a genetic dysmyelination disorders (Samsam *et al.* 2003), axonal dystrophy (Mi *et al.* 2005), autoimmune encephalomyelitis (a multiple sclerosis model :Kaneko *et al.* 2006), traumatic brain injury (Gillingwater *et al.* 2006a), glaucoma (Howell *et al.* 2007; Beirowski *et al.* 2008), Parkinson's diseases (Sajadi *et al.* 2004; Hasbani and O'Malley 2006), removal of nerve growth factor (NGF: Deckwerth and Johnson 1994) and CNS ischaemia-reperfusion injuries (Gillingwater *et al.* 2004). This would clearly suggest that the mechanisms that govern Wallerian degeneration in response to axotomy are important in mediating the loss of axons and nerve terminals in response to many neurodegenerative and toxic stimuli, and are linked by some shared cellular processes (Raff *et al.* 2002; Ferri *et al.* 2003; Coleman 2005; Luo and O'Leary 2005; Saxena and Caroni 2007). The *Wld^s* mutation has also helped to reveal that there is a second morphologically and mechanistically distinct process of nerve terminal loss: withdrawal (Parson *et al.* 1997; Gillingwater and Ribchester 2001; Gillingwater *et al.* 2002; Raff *et al.* 2002; Gillingwater *et al.* 2003; Parson *et al.* 2004; Luo and O'Leary 2005; Saxena and Caroni 2007; Murray *et al.* 2008b).

1.5.2 Withdrawal of nerve terminals

Structural Characterisation

While the *Wld^s* mutation protects α -motor axons and nerve terminals against axotomy-induced Wallerian degeneration, nerve terminal structure and function are only transiently protected for 3-7 days post axotomy (Tsao *et al.* 1994; Ribchester *et al.* 1995; Gillingwater *et al.* 2002; Gillingwater *et al.* 2003; Parson *et al.* 2004). Following this delay, nerve terminals undergo a process of asynchronous, distal to proximal retraction/‘withdrawal’ of their arborisation into the distal axon (Gillingwater and Ribchester 2001; Gillingwater *et al.* 2003; Parson *et al.* 2004). While there is some clustering of synaptic vesicles, neurofilaments and invasion of terminal Schwann cells, as in Wallerian degeneration, during withdrawal there is minimal disruption to the internal organization of nerve terminal boutons including little or no perturbations to mitochondria or fragmentation of axons or changes to the postsynaptic endplate (Gillingwater and Ribchester 2001; Gillingwater *et al.* 2003; Parson *et al.* 2004). Rather, nerve terminals are progressively and systematically retracted in a distal to proximal fashion into the pre-terminal axon that forms a bulbous retraction club until the nerve terminal has vacated its postsynaptic endplate. This process of withdrawal can be executed within 4hrs (Bettini *et al.* 2007) but is more typically seen over a period of days to weeks (e.g. Yin *et al.* 2004).

Mechanism of withdrawal

Like Wallerian degeneration the mechanisms that underlie the withdrawal of nerve terminals, particularly in adults, are still unknown (Bettini *et al.* 2007). The remarkably similar morphology between withdrawal and developmental synapse elimination suggests, at least in part, a shared mechanism (Bernstein and Lichtman 1999; Gillingwater *et al.* 2002; Raff *et al.* 2002; Gillingwater and Ribchester 2003; Parson *et al.* 2004), although upstream regulation appears to be different as the *Wld^s* mutation fails to alter developmental synapse elimination (Parson *et al.* 1997; Hoopfer *et al.* 2006). During developmental synapse elimination in α -motor neurons (Bixby 1981; Riley 1981; Keller-Peck *et al.* 2001; Walsh and Lichtman 2003, but compare Rosenthal and Taraskevich 1977) and post-injury re-innervation (Brown *et al.* 1976; Costanzo *et al.* 2000) muscle fibres that are poly-innervated, slowly adopt (over ~2 week period) the mature pattern of mono innervations, i.e., one motor neuron collateral innervating each muscle fibre, by removing exuberant innervation. As nerve terminals from different motor neuron collaterals compete for sole innervation the amount of endplate, which they occupy and alter their synaptic strength by expanding and contracting their arborisations (Colman *et al.* 1997; Herrera and Zeng 2003). The ‘losing’ nerve terminal is completely withdrawn into a retraction club and into the distal axon (Riley 1981). It has been suggested that the mechanisms that govern synapse elimination may be retained but modified or reactivated in adult peripheral nerve terminals as part of the repertoire of gross/ ongoing structural plasticity (Caroni 1997b). Small postsynaptic junction folds have been identified in rodents with no opposing presynaptic apparatus, also known as synaptic guttering, and is highly suggestive of recent structural reorganisation (e.g.,

Cardasis and Padykula 1981; Wernig *et al.* 1984; Wernig and Herrera 1986; Wigston 1989; 1990). This evidence of adult structural plasticity and the process of developmental synapse elimination in wild-type nerve terminals indicates that withdrawal of *Wld^s* nerve terminals is not a mechanism unique to the mutation. The cellular processes that govern withdrawal, especially in developmental, appear to be under the control of the actin cytoskeleton and myosin molecular motors (Wylie and Chantler 2003). Similar control is suggested in adults (Billuart *et al.* 2001; Luo and O'Leary 2005; Vega-Riveroll *et al.* 2005; Saxena and Caroni 2007) and indicates that withdrawal is an active process (c.f. Bernstein and Lichtman 1999). Interestingly, it has been shown (in adult *Drosophila*) that the cellular pathway that governs retraction is inhibited under normal physiological condition and that to initiate retraction the mechanism must be de-repressed (Billuart *et al.* 2001). Similar results have been found in rats (Lamprecht *et al.* 2002), and helps support the hypothesis that developmental processes and machinery for retraction are retained but modified in adults and are poised to act rapidly in response to stimuli (Luo and O'Leary 2005; Saxena and Caroni 2007).

Withdrawal in pathological processes

There are now increasing numbers of reports of nerve terminal withdrawal in response to pathological stimuli (Gillingwater *et al.* 2002; Gillingwater and Ribchester 2003). For example, in genetic dysmyelination disorders (Yin *et al.* 2004), progressive withdrawal of nerve terminals in mice over-expressing myelin specific Protein-0 caused >90% of nerve terminals to be partially or fully withdrawn by postnatal day 90. This preceded evidence of axonal degeneration that ultimately led to fatality by 6 months. Exogenous application of high affinity agonists to ATP-sensitive-P2X₇-purinoceptors known to be involved in neurodegeneration (Le Feuvre *et al.* 2002) has also been shown to cause progressive nerve terminal withdrawal akin to *Wld^s* withdrawal in de-nucleated preparations (Bettini *et al.* 2007). Much like Wallerian degeneration, this latter study shows that withdrawal can occur in the absence of cell bodies and therefore must be a mechanism that is under local control (Bettini *et al.* 2007). Withdrawal of nerve terminals has also been described in *wasted* mice (Murray *et al.* 2008b), following postsynaptic protein synthesis inhibition (McCann *et al.* 2007), postsynaptic blockade (Balice-Gordon and Lichtman 1994), application of neuregulin (a glial growth factor: Trachtenberg and Thompson 1997) and muscle fibre damage (Rich and Lichtman 1989; Bernstein and Lichtman 1999). It is however, the type of pathological stimuli that triggers withdrawal that has caused some disagreement as to whether withdrawal is an active or more passive process. These studies appear to show that withdrawal maybe triggered by loss/changes in trophic support supplied by neuronal protein synthesis (Murray *et al.* 2008b), cell bodies in *Wld^s* mice (Tsao *et al.* 1994; Ribchester *et al.* 1995; Gillingwater *et al.* 2002; Gillingwater *et al.* 2003; Parson *et al.* 2004), Schwann cells (Trachtenberg and Thompson 1997; Yin *et al.* 2004) or postsynaptic muscle fibres (Rich and Lichtman 1989; Balice-Gordon and Lichtman 1994; Bernstein and Lichtman 1999; McCann *et al.* 2007), and competition for trophic support is thought to be a key in mediating developmental synapse elimination (e.g., Bernstein and Lichtman 1999; Sanes and Lichtman 1999). This has led to the suggestion that withdrawal is the result of atrophy of nerve terminals due to loss of trophic support

(Bernstein and Lichtman 1999) indicating that it is a more passive process than perhaps the one mediated by the actin cytoskeleton suggested above. Nevertheless, what these studies primarily reveal is that there are at least two distinctive mechanisms of nerve terminal loss: withdrawal and Wallerian degeneration, and that they appear to be activated differentially depending on the type of stimulus.

1.5.3 Evidence of a third type of α -motor nerve terminal loss: necrosis

Although not as extensively studied, there is some evidence to suggest that very specific local insults to nerve terminals can cause a third type of nerve terminal loss: necrosis, that is characterised by the rapid breakdown of the presynaptic membrane. Following application of α -latrotoxin (LTX: black widow spider venom) or auto-ganglioside/complement attack (an experimental model of Miller-Fisher syndrome) that both selectively form large pores in the presynaptic membrane (Clark *et al.* 1970; Okamoto *et al.* 1971; Duchen *et al.* 1981; O'Hanlon *et al.* 2001; O'Hanlon *et al.* 2003; Ushkaryov *et al.* 2004), there is massive synaptic vesicle release, muscle contractures and functional block within about 15-60min. This is followed by bouton swelling, almost complete loss of synaptic vesicles, the cytoplasm becoming electron-lucent, breakdown of the cytoskeleton and presynaptic membrane, invasion of Schwann cell process and engulfment of the terminal boutons over the next 1-2hr. Both LTX and auto-ganglioside/complement attack are at least in part mediated by Ca^{2+} and proteolytic calpain activation (O'Hanlon *et al.* 2003). β -bungarotoxin⁴ also causes similar changes to α -motor nerve terminals as LTX and auto-ganglioside/complement attack. Following application of β -bungarotoxin there is rapid release of synaptic vesicles 0.5-3.5hr followed by irreversible functional block, loss of synaptic vesicles and their release machinery (synaptophysin, SNAP and syntaxin), swelling and lysis of mitochondria, and loss of neurofilaments over 3-12hr *in vivo* (Abe *et al.* 1976; Dixon and Harris 1999; Prasarnpun *et al.* 2004; Prasarnpun *et al.* 2005). This α -motor nerve terminal pathology appears to cause progressive retrograde fragmentation into pre-terminal axons and intramuscular nerve over the next 12hr. There is also breakdown of the presynaptic membrane and invasion of Schwann cell process and engulfment but unlike LTX and auto-ganglioside/complement, boutons become shrunken and cytoplasm becomes electron-dense. The mechanism of action of β -bungarotoxin is still unknown (Dixon and Harris 1999) and probably involves multiple molecular mechanisms including phospholipid hydrolysis, massive Ca^{2+} entry and a direct action on mitochondria and opening of their pores (Prasarnpun *et al.* 2004; Rigoni *et al.* 2008).

⁴ β -bungarotoxin is a specific pre-synaptic neurotoxin and has a very different action from α -bungarotoxin that has a post-synaptic action by binding to AChRs (that latter has been used extensively in the current study). Both however, form part of the lethal venom of the *Bungarus* genus of snakes that are widespread over southeast Asia. The toxins were originally identified by C. Chang and colleagues during the 1960-70's in the Taiwanese banded krait (*B. multicinctus*: Chang 1999)

1.5.4 Compartmentalisation & Nerve terminal vulnerability

As discussed earlier, Wallerian degeneration of axons and nerve terminals, and apoptosis of cell bodies are not mechanistically linked. Since the discovery of the *Wld^s* mutation and with further study, it has become apparent that there are distinct degenerative mechanisms relating to each neuronal compartment (Gillingwater and Ribchester 2001; Gillingwater *et al.* 2002; Raff *et al.* 2002; Coleman 2005; Wishart *et al.* 2006; Conforti *et al.* 2007a). In cultured superior cervical ganglion removal of NGF in wild-type neurons normally results in apoptosis of cell bodies and degeneration of axons but if the *Wld^s* protein is present, removal of NGF results in loss of only the cell bodies while axons survive (Deckwerth and Johnson 1994). Similarly, the *Wld^s* mutation does not prevent the death of cell bodies in young rats following axotomy (Adalbert *et al.* 2006) or the loss of cell bodies in experimental models of glaucoma (Beirowski *et al.* 2008) and alternatively, protecting cell bodies from apoptosis by over-expression of Bcl-2 protein does not confer protection to axons (Sagot *et al.* 1995; Burne *et al.* 1996). This evidence collectively suggests that there is compartmentalisation of degenerative mechanisms and that they may be activated independently by different stimuli (Gillingwater and Ribchester 2001; Raff *et al.* 2002; Gillingwater and Ribchester 2003; Wishart *et al.* 2006): apoptosis in cell bodies, Wallerian degeneration in axons, and at least two mechanisms in nerve terminals: Wallerian degeneration and withdrawal. This concept of compartmentalisation is particularly important when studying neuropathogenesis (Gillingwater and Ribchester 2001; 2003; Coleman 2005; Wishart *et al.* 2006). To help prevent the manifestation of punitive symptoms it is vital to determine where pathology begins (i.e., in which compartment) and then direct therapeutic strategies to that compartment to prevent loss of connectivity, the ultimate cause of the clinical and punitive symptoms (Wishart *et al.* 2006; Conforti *et al.* 2007a; Kariya *et al.* 2009). Examination of the early events of neuropathological conditions has provided overwhelming evidence that indicates nerve terminals are the principal site of structural and functional loss (see Table 1) and include many of the most prevalent conditions affecting the modern world. Further compelling evidence for compartmentalisation and vulnerability of nerve terminal is provided by studies into amyotrophic lateral sclerosis (ALS). ALS is 'dying back' neuropathy that progresses in a distal to proximal manner with nerve terminal loss identified as a principal event, and in familiar models of ALS this is caused by mutations in the superoxide dismutase 1 (mSOD1) gene (e.g., Fischer *et al.* 2004 and Wishart *et al.* 2006, see Table 1). When axons are protected by the *Wld^s* mutation in mice expressing mSOD1 there is little or no improvement in animals survival, loss of nerve terminals or the disease's punitive effects (Vande Velde *et al.* 2004; Fischer *et al.* 2005). Similarly, protection of α -motor neuron cell bodies in mSOD1 mice with glial derived neurotrophic factor does not prevent loss of α -motor nerve terminals or improve the symptoms of the disease (Suzuki *et al.* 2007). Similar results have been documented in mouse models of spinal muscular atrophy where the *Wld^s* mutation failed to prevent nerve terminal loss and the diseases punitive affects (Kariya *et al.* 2009), in hereditary spastic paraplegia (Edgar 2004) that has also been identified as a 'dying back' neuropathy (Ferreirinha *et al.* 2004) and following disruption of CNS nerve terminals with botulinum C neurotoxin that also triggers

withdrawal (Berliocchi *et al.* 2005). This clearly indicates that nerve terminals are highly vulnerable and highlights the need to identify the correct neuronal compartment and cellular mechanism for effective neuroprotection.

1.5.5 Summary

In summary, I have reviewed some of the evidence that indicates neurons have compartmentalised responses to different pathological stimuli and that overall, nerve terminals are highly vulnerable with functional and structural loss triggered by a range of toxic, traumatic and neurodegenerative disease stimuli. This is particularly evident in α -motor nerve terminals, where following axotomy the first morphological signs of Wallerian degeneration are identified in α -motor nerve terminals, and even when axotomy induced Wallerian degeneration is inhibited by the *Wld^s* mutation, nerve terminals are still only transiently protected for a few days before being lost by withdrawal. Based on this general concept of nerve terminal vulnerability, it would not be surprising if α -motor nerve terminals were also vulnerable to hypoxia. The precedence for this can be observed in the CNS in response to ischaemia and metabolic inhibition (see Table 1) and is discussed in the next section.

Table 1: Traumatic, toxic and neurodegenerative disease stimuli identified with nerve terminal loss as principal event and/or causing ‘dying back’ neuropathies.

Disruption of microtubule network (Vincristine neuropathies)	Hsu and Lentz 1972; Wang <i>et al.</i> 2000; Wang <i>et al.</i> 2001
gracile axonal dystrophy	Miura <i>et al.</i> 1993; Mi <i>et al.</i> 2005
spinal muscular atrophy	Cifuentes-Diaz <i>et al.</i> 2002; Murray <i>et al.</i> 2008a; Kariya <i>et al.</i> 2009
Mechanical Trauma/Axotomy	Birks <i>et al.</i> 1960; Miledi and Slater 1968; 1970; DeSantis and Norman 1979
Charcot-Marie-Tooth Disease & Demyelination	Samsam <i>et al.</i> 2003; Yin <i>et al.</i> 2004
Bachtrachotoxin	Hudson <i>et al.</i> 1984
Guillane-Barre syndromes (including Miller-Fisher syndrome)	Roberts <i>et al.</i> 1994; O’Hanlon <i>et al.</i> 2001; Spaans <i>et al.</i> 2003; Goodfellow <i>et al.</i> 2005
Focal postsynaptic blockade	Balice-Gordon and Lichtman 1994
beta-bungarotoxin	Abe <i>et al.</i> 1976; Dixon and Harris 1999; Prasarnpun <i>et al.</i> 2004; Prasarnpun <i>et al.</i> 2005
P2X ₇ -receptor activation	Bettini <i>et al.</i> 2007
Myasthenia gravis	Rich <i>et al.</i> 1994
Dynein/dynactin molecular motor disruption	LaMonte <i>et al.</i> 2002; Hafezparast <i>et al.</i> 2003; Laird <i>et al.</i> 2008
Muscle fibre trauma	Rich and Lichtman 1989; Bernstein and Lichtman 1999
Alcoholic neuropathy	Casey and Le Quesne 1972
deletion of translation factor eEF1A2 (Wasted mice)	Newbery <i>et al.</i> 2005; Murray <i>et al.</i> 2008b
Organophosphate toxicity	Prineas 1969b; Glazer <i>et al.</i> 1978; Kawabuchi <i>et al.</i> 1991
Motor neuron disease	Schmalbruch <i>et al.</i> 1991b; Frey <i>et al.</i> 2000; Ferri <i>et al.</i> 2003
Latrotoxin	Clark <i>et al.</i> 1970; O’Hanlon <i>et al.</i> 2003
Zinc pyrithione toxicity	Sahenk and Mendell 1979
Vacor (rodenticide) toxicity	Watson and Griffin 1987
dithiobiuret toxicity	Kemplay 1984; Sahenk 1990
Intoxication by the fruit of the tullidora shrub	Munoz-Martinez <i>et al.</i> 1983
Botulinum toxin	Pastor <i>et al.</i> 1997; Berliocchi <i>et al.</i> 2005
Critical illness polyneuropathy	Schwarz <i>et al.</i> 1997
Inhibition of post-synaptic protein synthesis	McCann <i>et al.</i> 2007
Diabetic neuropathy	Fahim <i>et al.</i> 2000; Dobretsov <i>et al.</i> 2007; Ramji <i>et al.</i> 2007; Muller <i>et al.</i> 2008
Temporary limb immobilisation & space flight	Mohamed 1989; Riley <i>et al.</i> 1990; D’Amelio and Dauntun 1992
Palytoxin	Amir <i>et al.</i> 1997
Paralysis induced by upper spinal injury	Burns <i>et al.</i> 2007a
Chronic corticosterone treatment	Mohamed 1995
Normal ageing (cognitive, motor & sensory decline)	Swash and Fox 1972; Cupp and Uemura 1980; Fahim and Robbins 1982; Larsson and Ansved 1995; Tanaka <i>et al.</i> 1996; Peters <i>et al.</i> 1998
Hereditary spastic paraplegia	Ferreirinha <i>et al.</i> 2004
acrylamide poisoning	Fullerton and Barnes 1966; Fullerton 1969; Prineas 1969a; LoPachin <i>et al.</i> 2002
Traumatic brain injury	Erb and Povlishock 1991; Gillingwater <i>et al.</i> 2006a
Use of methamphetamine (aka ‘crystal meth’ or speed)	Cadet <i>et al.</i> 2003
Huntington’s disease	Graveland <i>et al.</i> 1985; DiProspero <i>et al.</i> 2004; Ribchester <i>et al.</i> 2004; Milnerwood <i>et al.</i> 2006
amyotrophic lateral sclerosis (ALS) & mSOD1	Sasaki and Iwata 1996; 1999; Frey <i>et al.</i> 2000; Fischer <i>et al.</i> 2004; Schaefer <i>et al.</i> 2005; Zang da <i>et al.</i> 2005
Prion disease & spongiform encephalopathies	Kitamoto <i>et al.</i> 1992; Clinton <i>et al.</i> 1993; Niewiadomska <i>et al.</i> 2002; Cunningham <i>et al.</i> 2003
Human immunodeficiency virus (HIV)-associated dementia	Kim <i>et al.</i> 2008
Thrombin injection	Herrera <i>et al.</i> 2008
Vitamin E deficiency	Southam <i>et al.</i> 1991
Deficiency in dietary protein intake (kwashiorkor)	Oldfors 1981; Oldfors and Persson 1982
Succinate dehydrogenase inhibition	Sonsalla <i>et al.</i> 1997
Alzheimer’s Disease	Davies <i>et al.</i> 1987; Terry <i>et al.</i> 1991; Coleman <i>et al.</i> 2004
Cathepsin D deficiency	Partanen <i>et al.</i> 2008
Ischaemia-reperfusion injury (CNS)	Williams and Grossman 1970; Rothman 1983; Ishimaru <i>et al.</i> 2001; Jourdain <i>et al.</i> 2002; Kovalenko <i>et al.</i> 2006

1.6 The vulnerability of CNS nerve terminals to hypoxia & ischaemia-reperfusion injury

Studies into hypoxia/ischaemic injuries, ‘stroke’ or ‘cerebral vascular accident’ has a long history with Hippocrates (460-370 BC) being one of the first to describe the sudden onset and paralysis caused by ‘apoplexy’ or transient ischaemia (Thompson 1996). It is now known that even a brief hypoxic/ischaemia insult in the CNS will cause significant loss of neurons leading to either death of the patient or long-term brain damage and disability. Perhaps because ischaemic brain injury is one of the leading causes of disability and mortality worldwide (Dewar *et al.* 1999; White *et al.* 2000), it is also one of the most extensively studied. In the following section, I will briefly discuss some of the mechanisms of neuronal cell death in response to ischaemic/hypoxic insults as a basis for demonstrating the vulnerability of CNS nerve terminals and for understanding potential events at peripheral α -motor nerve terminals.

1.6.1 Ischaemia-reperfusion injuries trigger a complex maelstrom of pathological stimuli

Ischaemia refers to the reduction or loss of the blood supply and it is generally agreed that the major pathological stimulus brought about by ischaemia is the lack of oxygen (hypoxia) that inhibits oxidative phosphorylation causing ATP depletion (Dewar *et al.* 1999; Lipton 1999; Ames 2000; White *et al.* 2000; Bickler and Donohoe 2002). Unlike skeletal muscle or the liver, neurons do not store significant amounts of chemical fuel stores to supplement ATP supply when either O_2 or glucose is lacking, making them highly vulnerable to hypoxic/ischaemic insults (Zubay 1998; Ames 2000; Attwell and Laughlin 2001). Precipitous falls in ATP levels are observed within minutes of the onset of *in vivo* ischaemia and *in vitro* hypoxia and this triggers ionic gradient breakdown leading to a rise in extracellular K^+ and intracellular Na^+ followed by a rise in intracellular Ca^{2+} (e.g., Silver 1977; Lipton 1999; Ames 2000). The mechanism of this ionic gradient breakdown is still unclear and many authors have attributed it to failure of the Na^+/K^+ ATPase pump to which so much of the neuron’s energy supplies are devoted, but while this seem likely, it has yet to be conclusively shown (Lipton 1999). The rise in intracellular Ca^{2+} has been attributed to disruption of Ca^{2+} stores and in particular mitochondria, but also reversal of the Na^+/Ca^{2+} exchanger in response to elevated Na^+ and opening of voltage dependent Ca^{2+} channels (Lobner and Lipton 1993; Zhang and Lipton 1999; Jeffs *et al.* 2007). This ionic gradient breakdown triggers release of neurotransmitters, in particular glutamate from nerve terminals to activate post-synaptic NMDA⁵ receptors, exacerbating Ca^{2+} entry and triggering excitotoxicity (Olney 1969; Rothman 1983; Benveniste *et al.* 1984; Rothman 1984; Choi 1988;

⁵ NMDA: *N*-methyl-d-aspartate,

Lobner and Lipton 1993; Zhang and Lipton 1999). Excitotoxicity and many of these hypoxic/ischaemic events may trigger necrotic cell death, as the elevated Ca^{2+} can be sufficient to trigger proteolytic calpains (Siesjo 1988), while progressive inhibition of electron transport, mitochondrial swelling and/or activation of mitochondrial pore opening due to ionic imbalance may activate apoptotic cell death via the release of cytochrome *c* from the mitochondria (e.g., Lipton 1999; White *et al.* 2000). Ischaemia is however associated with a range of potential pathological stimuli, not just hypoxia, that may also contribute to injury. For example, ischaemia causes significant intracellular and extracellular acidification (Silver 1977; Kass and Lipton 1982; Fujiwara *et al.* 1992) that if prevented, by maintaining pH with an alkaline buffer, can reduce the area of cell death following *in vivo* ischaemia in the cat by ~40% (Nagao *et al.* 1996). The underlying cause of this acidification is still unknown (Obara *et al.* 2008) but possible mechanisms including reversal of Na^+/H^+ transporters in response to elevated levels of Na^+ , increasing dephosphorylation of ATP that releases H^+ ions and/or disruption of the bicarbonate buffering system coupled to increasing CO_2 levels have been suggested (Lipton 1999; Cerretelli and Samaja 2003; Obara *et al.* 2008). Ischaemia also causes a fall in glucose levels, maintenance of which is vital for continuing respiration via glycolysis that may be able to help support ATP levels but is also associated with lactate production and increasing acidification (Ridge 1972; Lipton 1999; Cerretelli and Samaja 2003; Malthankar-Phatak *et al.* 2008). There is also accumulation of other metabolic by-products such as free fatty acids and hypoxanthine, the latter from the breakdown of adenosine nucleotides (Gardiner *et al.* 1981; Zhang 1995; Abramov *et al.* 2007). Reoxygenation or reperfusion, while vital for tissue survival, may also cause cellular damage and is the reason why ischaemia/hypoxia insults are normally referred to as hypoxia/ischaemia-reperfusion injuries. They are typically associated with systemic inflammation and the formation of reactive oxygen species (ROS) which can uncontrollably react with and damage cellular structures and cause inactivation of receptors, enzymes and increased leakiness of cell membranes (Halliwell 2006). With returning oxygen levels ROS may be produced (Chan 1996) directly from damaged mitochondrial electron transport chains (Piantadosi and Zhang 1996; Nicholls and Budd 2000) and by clearance of accumulated metabolic by-products such as hypoxanthine by xanthine oxidase (Abramov *et al.* 2007) as well as fatty acids (Chan and Fishman 1980; White *et al.* 2000) and reactions with Fe^{2+} delocalized from intracellular stores (Komara *et al.* 1986). Infiltration and up-regulation of leukocytes, cytokines and other inflammatory mediators such as nitric oxide synthase and cyclooxygenase, while an important injury response, may also contribute to damage by triggering ROS formation and other damaging stimuli (Nogawa *et al.* 1997; Samdani *et al.* 1997; Fukuyama *et al.* 1998; Lipton 1999; Costa *et al.* 2006; Doyle *et al.* 2008; Froyland *et al.* 2008). Reperfusion is also the stage where most cell death occurs (delayed cell death), when neurons that appear to survive the initial ischaemic insult die hours to weeks later due to propagation and accumulation of cell damage (e.g. Nakano *et al.* 1990; Lipton 1999). For example, Kovalenko *et al.* (2006) observed no signs of cell death in rat hippocampal neurons up to 2hr after a 15min ischaemic insult induced by clamping of the carotid artery, however by 7 days, 64% of neurons were undergoing necrosis and a further 16% showed some degenerative changes.

1.6.2 The role of nerve terminals in ischaemia-reperfusion injuries

Overall, ischaemia-reperfusion injuries cause a maelstrom of biochemical and cellular events that we are only just beginning to understand, but it is becoming increasingly clear that the events at nerve terminals have a key role in many of these pathological responses. The best characterized of these events is the release of glutamate from nerve terminals to trigger excitotoxicity. This was demonstrated in the early 1980's when primary cultures of rat hippocampal neurons maintained in an hypoxic environment (95% N₂: 5% CO₂) become swollen and vacuolated within a few hours and died by 14hr. Blocking transmitter release (Rothman 1983) or inhibiting the post-synaptic action of glutamate Rothman (1984) attenuated neuronal cell death and provided the first clear evidence that hypoxia was capable of causing neuronal cell death via action at nerve terminals. We also know that as well as initiating excitotoxic events in neighbouring neurons, functional and structural changes to nerve terminals are one of the first pathological events to occur in the parent neuron. Rats subject to 1 hour of middle cerebral artery occlusion, a relatively mild ischaemic insult that causes delayed cell death, followed by 1-3hr reperfusion have been shown to partially recover to propagate action potentials but display no postsynaptic potentials, suggesting long term functional failure of nerve terminals Bolay *et al.* (2002). Further investigation by Bolay *et al.* (2002) showed that phosphorylation of synapsin-I, involved in mobilisation of synaptic vesicles towards active zones, was significantly decreased. Others have also shown similar selective loss of critical vesicular proteins during reperfusion following mild ischaemic insult including SNAP-25, synaptophysin and mint1 prior to whole cell death (Nishimura *et al.* 2000; Ishimaru *et al.* 2001). Kovalenko *et al.* (2006) also noted no degenerative changes to cell bodies prior to 24hr after 15min ischaemic insult in rat hippocampus but observed widening of the synaptic cleft, decreases in the number of synaptic vesicles while those that remained were clustered away from the membrane after 2hr reperfusion. These structural changes at nerve terminals were followed by whole cell death after 3-7days of reperfusion and only those neurons (~20%) that presented with no alterations in nerve terminal ultrastructural appeared to survive beyond 7 days. Similar results have been obtained by others, showing progressive loss of rat hippocampal synaptic vesicles beginning within 10min of ischemia (von Lubitz and Diemer 1983). In cat cerebro-cortical synapse rendered ischaemic by chronic hypotension, electrodes confirmed rapid depolarisation within 5min and ultrastructural studies showed clumping and retraction of synaptic vesicles from the presynaptic membrane, increased frequency of nerve terminal profiles with no vesicles and all in advance of other structural changes (Williams and Grossman 1970). Others have also demonstrated selective degenerative changes in cultured rodent hippocampal slices subjected to environmental hypoxia and glucose deprivation (the accepted *in vitro* model of ischaemia: Jourdain *et al.* 2002; Jung *et al.* 2004; Kovalenko *et al.* 2006) but as is typical of these cultured model systems, it required a longer period of ischaemia compared to *in vivo* models to induce pathological changes (Lipton 1999; Kovalenko *et al.* 2006). Collectively, this has led to the suggestion that signalling events at nerve terminals and/or nerve terminal loss propagates back towards the cell body to instigate cell death (Martone *et al.* 1999; Nishimura *et al.* 2000; Ishimaru *et*

al. 2001; Bolay *et al.* 2002; Jourdain *et al.* 2002). What the pathological stimulus is or stimuli are that are responsible for loss of nerve terminals are unknown, however direct inhibition of nerve terminal metabolism alone has been shown to cause retrograde neuronal death. Microinjection of malonate, a SDH inhibitor, into rat striatum where dopaminergic neurons project, caused loss of their cell bodies in the substantia nigra 7 days later, which clearly indicates that inhibition of electron transport can cause loss of nerve terminals and retrograde cell death (Sonsalla *et al.* 1997; Zeevalk *et al.* 1997).

1.6.3 Summary

I have previously discussed that neurons appear to be highly compartmentalised in terms of anatomy, function, metabolism, and vulnerability to pathological stimuli and put forward the hypothesis that α -motor nerve terminals are probably highly vulnerable to hypoxic/ischaemic insults. Here I have discussed some of evidence that shows a precedence for nerve terminal vulnerability to hypoxic/ischaemic-reperfusion injuries in the CNS. By utilising a range of different *in vivo* and *ex vivo* model systems, the complex events of hypoxia/ischaemia-reperfusion injuries in the CNS appears to be largely initiated by metabolic failure at nerve terminals. Due to lack of ATP, progressive depolarisation causes excitotoxic cell death in neighbouring neurons while in the parent neuron, other functional and structural changes to nerve terminals appears to lead to cell death. Despite our increasing understanding of the events that lead to loss of nerve terminals and neuronal death in the CNS following hypoxia/ischaemia-reperfusion injuries, and the precedent this sets, very little is known about the response of α -motor neurons and their nerve terminals in the PNS.

1.7 Are α -motor nerve terminals vulnerable to hypoxia-reperfusion injury?

In the following section, I will discuss that while there is some evidence to indicate that α -motor nerve terminals of the PNS are highly vulnerable to hypoxia/ischaemia-reperfusion injuries, most of these studies are inconclusive. Due the fact that the PNS can be extremely susceptible to such injuries, especially as the result of application of surgical and non-surgical tourniquets, establishing that the vulnerability of α -motor nerve terminals to hypoxia/ischaemia-reperfusion injuries is of significant importance and worthy of further study.

1.7.1 The susceptibility of the PNS to hypoxia/ischaemia-reperfusion injuries

When some of the metabolic properties of neurons from the CNS and PNS are compared, it appears that the PNS has a greater metabolic safety factor than the CNS, suggesting an increased resistance to hypoxia/ischaemia. Peripheral nerves have been estimated to have 10% of the brain's oxygen requirements, but similar ATP levels (Stewart *et al.* 1965; Low *et al.* 1985; Zollman *et al.* 1991) and they also appear better able to supplement their energy requirements via anaerobic pathways (Campa and Engel 1971; Low *et al.* 1985; Mohler *et al.* 1999). The PNS however, is not invulnerable to hypoxic/ischaemic insults and it is perhaps their susceptibility to hypoxia/ischaemia that drives the requirement for a high metabolic safety factor. Unlike the CNS, blood flow to peripheral nerves is not auto-regulated and reductions in blood pressure result in reductions in neuronal blood supply (Smith *et al.* 1977; Low and Tuck 1984). Consistent with the idea of α -motor neurons being compartmentalised, this lack of auto-regulation makes peripheral axons and nerve terminals highly susceptible to reductions in oxygen supply compared to cell bodies located in the spinal cord. Reduced oxygen delivery in the extremities can therefore be expected in cold environments when blood flow is reduced to maintain core temperature, intermittent blood flow in resting muscle (Shibata *et al.* 2005), quick rest-to-work transitions and lateral forces produced during muscle contraction may all cause temporary ischaemia and deficits in oxygen supply (Kernell 2006; Clanton 2007). Poor gaseous exchange in the lungs during chronic obstructive pulmonary disease, ageing, and low oxygen environments may also produce decreases in systemic oxygen levels. Cardiovascular diseases, haematological disorders that affect the oxygen binding/release capacity of haemoglobin, thromboses and traumatic injury are also other clinical conditions that may cause a significant reduction in blood supply and oxygen delivery to peripheral tissues. In particular, the routine use of surgical and non-surgical tourniquets to control bleeding, blood flow, blood pressure and for the regional administration of drug therapies will render entire limbs ischaemic for hours at a time (McEwen 1981; Idstrom *et al.* 1990; Pedowitz *et al.* 1992; Kam *et al.* 2001; Kam 2007; Murphy and O'Connor 2007). It is such acute

ischaemia-reperfusion injuries caused by the application of tourniquets that are of most interest and form the focus of the following discussion and study.

1.7.2 Long-term neurological damage caused by tourniquets

Tourniquets have a long history, with the first recorded use in the second century AD by the Roman surgeon Heliodorus, with the French surgeon Jean Louis Petit giving the name 'tourniquet' ('*tourner*' meaning '*to turn*') in 1718 to his screw device that could stem blood flow (Leonardo 1943; Rorabeck 1980; Murphy and O'Connor 2007). This was followed over a century later by the von Esmarch's elastic bandage in 1873 and the development of the pneumatic tourniquet by Harvey Cushing c.1904 upon which all modern, computer controlled, tourniquets are based (Rorabeck 1980; McGraw and McEwen 1987; Murphy and O'Connor 2007). Tourniquets are a vital part of everyday clinical practice but are well known to cause potentially severe injuries with von Esmarch being one of the first to caution the use of his own 'Esmarch' bandages in 1873:

“...we must not ignore the possibility that the firm strapping of a limb for any considerable length of time may be followed by dangerous derangements of the circulation and innervation such as thrombosis, inflammation, paralysis, etc.”

(Von Esmarch 1873, quote taken from Rorabeck 1980)

Occluding the blood supply to an entire limb or multiple limbs (by either tourniquets or vascular occlusion) over a long time frame is a massive undertaking (Rorabeck 1980) that can lead to amputations and even in the clinical setting, death (Chervu *et al.* 1989; Carden and Granger 2000; Kam *et al.* 2001). Much like ischaemia in the CNS, ischaemia in peripheral limbs will also cause hypoxia, reduced glucose, acidosis, increased levels of lactate and other metabolites, CO₂, K⁺, Ca²⁺, and oedema with the sheer mass of tissues probably exaggerating these changes. When blood flow is restored (which may be delayed due to vascular collapse: Ames *et al.* 1968; Gustafsson *et al.* 1999) there is a systemic rise in K⁺, Ca²⁺, ROS generation especially from xanthine oxidase, and systemic acidosis that can all perpetuate local tissue damage but also trigger compartment syndrome, systemic inflammatory response, shock, and multiple organ dysfunction (Silver 1977; Rorabeck 1980; McGraw and McEwen 1987; Idstrom *et al.* 1990; Pedowitz *et al.* 1992; Nagamatsu *et al.* 1996; Gustafsson *et al.* 1999; He *et al.* 1999; Mitsui *et al.* 1999a; Carden and Granger 2000; Kam *et al.* 2001; Matsumoto *et al.* 2005; Bagdatoglu *et al.* 2006; Kam 2007; Bagdatoglu *et al.* 2008). Tourniquets are most clearly associated with skeletal muscle damage and necrosis both underneath and distal to the tourniquet. Numerous authors have reported perturbations and loss of muscle fibre striations, whole muscle fibres becoming wavy, vacuolised, swollen or atrophied with disrupted cell membranes leading to necrotic muscle fibre death following as little as 2hr of ischaemia (Scully *et al.* 1961; Stenger *et al.* 1962; Haljamae and Enger 1975; Makitie and Teravainen 1977a; Patterson and Klenerman 1979; Mars and Gregory 1991; Pedowitz *et al.* 1992; Awerbuck *et al.* 1994; Ohara *et al.* 1996; Woitaske and McCarter 1998; Mohler *et al.* 1999; Kawamura *et al.* 2006). For this reason the upper limit of applied time for

surgical tourniquets has generally been agreed to be 1.5-2hr (e.g. McElvenny 1945; Kessler 1966; Rorabeck 1980; Pedowitz *et al.* 1992; Kalla *et al.* 2003; Younger *et al.* 2005; Malanjam and Fischer 2006; AORN 2007; Kam 2007), although some have suggested that skeletal muscle may survive total ischaemia for as long as 4hr (Gustafsson *et al.* 1999). In spite of this ~2hr upper limit, there are numerous reports of paralysis and long term motor deficits following peripheral limb ischaemia. In human surgical patients the most common complications following tourniquet use are neurological and range from mild functional loss through to complete limb paralysis and permanent functional deficits (e.g., Bernard Roth 1931; Eckhoff 1931; McElvenny 1945; Rudge 1974; Saunders *et al.* 1979; Rorabeck 1980; McGraw and McEwen 1987; Kalla *et al.* 2003; Kam 2007; Murphy and O'Connor 2007). This has been estimated to affect >60% of surgical patients (Saunders *et al.* 1979), or up to 1:11,000 and 1:250,000 of the UK population following the use of surgical tourniquets on the upper and lower limbs respectively (Kam 2007) but under-reporting may mean that the incidence of ischaemic tourniquet paralysis is much higher (Kam *et al.* 2001). Similar long term functional and behavioural deficits (e.g. reduced grip, toe spreading, self-mutilation etc) have been observed in animal models following as little as 2hr ischaemia which tend to worsen over time/during reperfusion (e.g., Fowler *et al.* 1972; Makitie and Teravainen 1977a; b; Nitz *et al.* 1986; Zollman *et al.* 1991; Pedowitz *et al.* 1992; Iida *et al.* 2003; Wang *et al.* 2008). For example, after 4hr arterial occlusion in adult rats or 3hr of tourniquet-induced ischaemia in adult baboons, the ability to elicit muscle action potentials was still significantly reduced and movement of limbs impaired after 42 and 180 days reperfusion respectively (Fowler *et al.* 1972; Iida *et al.* 2003). This would suggest that PNS is highly vulnerable to ischaemia-reperfusion injuries and results in punitive and long-term neuropathies. With an estimated ~1million procedures utilising surgical and non-surgical tourniquets annually in the USA (McEwen 1981), and the average orthopaedic surgeon applying ~200 tourniquets per annum (Middleton and Varian 1974), a clear understanding of what is causing these long term injuries is of significant clinical importance.

1.7.3 Effects of hypoxia/ischaemia-reperfusion injuries on peripheral nerves.

One of the first reports of peripheral ischaemic neuropathy was made by Thomas (1909), who reviewed numerous case reports of his predecessors, peers, and his own patients regarding 'ischaemic paralysis'. He concluded that in ~60% of patients that had splints, bandages and plaster casts applied to their limbs, paralysis could be directly attributable to nerve damage and that this is most likely the result of disturbances in the circulation. We now know that when a peripheral nerve is rendered ischaemic either by arterial occlusion or by application of tourniquets, there is a failure to transmit action potentials or 'conduction block' within ~35mins (Gerard 1930; Maruhashi and Wright 1967; Low *et al.* 1985; Zollman *et al.* 1991). During vascular occlusion in rats, a reduction in caudal nerve action potential amplitude was seen after 10min and complete conduction block within 35mins which coincided with a 50% decrease in ATP levels by 10mins that dropped to less than 10% after 60mins (Low *et al.* 1985). Both the axonal conduction block and metabolic changes can be reversed within

3hr reperfusion following mild ischaemic insults (Low *et al.* 1985; Zollman *et al.* 1991). This clearly indicates metabolic failure in response to ischaemia and is likely to underlie membrane depolarisation (Maruhashi and Wright 1967; Low *et al.* 1985), in keeping with the high energetic demands of maintaining and re-establishing polarised membranes (see section 1.4.1). More severe ischaemia causes structural changes to sciatic/tibial nerves underlying and distal to tourniquets with minor axonal swelling and degenerative changes after 3hr ischaemia alone, followed by clear signs of lesions, degeneration and demyelination following reperfusion (e.g. Fowler *et al.* 1972; Makitie and Teravainen 1977b; Nukada and McMorran 1994). In vascular occlusion studies similar findings have been noted, where minor degenerative changes and swelling have been noted after 3hr (Iida *et al.* 2003; Kawamura *et al.* 2006) with obvious signs of degeneration after 7 days reperfusion (Nukada and McMorran 1994; Iida *et al.* 2003; Kawamura *et al.* 2006). These studies have led to the suggestion that reperfusion is a major factor in causing neuronal injury (Nukada and McMorran 1994; Iida *et al.* 2003), with significant inflammatory response including infiltration of inflammatory cells and cytokines (Mitsui *et al.* 1999c; Kawamura *et al.* 2006), nitric oxide (Qi *et al.* 2001) and ROS especially from xanthine oxidase leading to oxidative damage of proteins (Nagamatsu *et al.* 1996; He *et al.* 1999; Mitsui *et al.* 1999a). Overall, this is consistent with some of the biochemical and mechanistic events known to occur in CNS hypoxia/ischaemia-reperfusion injuries but unlike the CNS, the potential involvement of nerve terminals in such injuries has been neglected or overlooked. This is a critical issue to resolve as by not clearly examining possible structural and functional events at α -motor nerve terminals may have led to incorrectly identifying the primary target of hypoxia/ischaemia-reperfusion injuries as being skeletal muscle whose tolerance to hypoxia/ischaemia-reperfusion injuries determines the applied time of tourniquets.

1.7.4 Are α -motor nerve terminals vulnerable to ischaemia-reperfusion injuries?

In his 1909 paper describing ischaemic paralysis, Thomas also discusses the work of the German clinician, Chvostek, who published a case report in 1892 (Chvostek 1892) describing a patient who presented with flaccid paralysis in his right leg and was found at autopsy to have an embolism in his popliteal artery. Chvostek apparently argued that the paralysis in the first instance was due to damage of the nerve endings but Thomas (1909) largely rejected this view, saying the affects of “terminal muscle branches” was of secondary consequence to muscle atrophy and degeneration in response to ischaemia. This viewpoint still dominates even in modern clinical practice and in experimental models. It is typical practice for neuromuscular function to be assessed by measuring muscle action potentials (MAP) generated by stimulating the distal nerve (Iida *et al.* 2003). While this method may give an indication of overall functional deficits, it is inappropriate for distinguishing neurological deficits from that of skeletal muscle and may have led to widespread misinterpretation of data (Eastlack *et al.* 2004). For example, Zollman *et al.* (1991) have shown that 30mins reperfusion after

1hr arterial occlusion is sufficient to restore nerve action potential propagation measured between two electrodes on the rat sciatic/tibial nerve. MAP measured in the muscles of the foot upon stimulation of sciatic/tibial nerve at the ankle however, remained less than 34% of control values 1 week after reperfusion and if ischaemia was increased to 3hr, values are less than 30% of control values after 1month reperfusion. On the basis of this experimental evidence, Zollman *et al* (1991) and others using similar techniques (e.g., Fish *et al.* 1989; Pedowitz *et al.* 1992; Ohara *et al.* 1996; Mohler *et al.* 1999) have largely concluded that the primary site of ischaemia-reperfusion injury is skeletal muscle but their results could equally reflect transmission failure at the NMJ rather than in axons or skeletal muscle. A recent study has shown clear functional evidence that deficits occur in the nerve before the muscle (Hatzipantelis *et al.* 2001). Ischaemia induced by arterial occlusion in rats produced a rapid and progressive decrease in muscle fibre contractile properties and at 50-80min there was complete failure to elicit muscle fibre contraction via stimulation of the nerve. This reduction in contractile force was almost completely reversed when muscles were stimulated directly, independently of the nerve supply. This study is supported by other studies in the rabbit following vascular occlusion for 1-3hr (Chervu *et al.* 1989) and has also been shown 24hr after tourniquet ischaemia in rats (Eastlack *et al.* 2004: c.f discussion below ,section 1.7.5). These studies clearly identify that ischaemia-reperfusion injury affects nerve more severely than skeletal muscle and that the principal site of injury is at the NMJ and most likely at α -motor nerve terminals (Eastlack *et al.* 2004). In the former of these studies, Hatzipantelis *et al* (2001) go on to show that after 50-80min ischaemia induced by arterial occlusion and cessation of neuromuscular transmission, significant degenerative changes could be observed in α -motor nerve terminals. Makitie & Teravainen (1977b) have also shown swelling of mitochondria and some minor degenerative changes in rat α -motor nerve terminals but these changes were not always independent of minor postsynaptic or skeletal muscle pathology. Similar findings were found following transection of the vascular supply (carefully avoiding the nerve supply) in rat soleus muscles where some muscle fibre pathology was observed alongside both IA/II, γ - (the nervous supply of the muscle spindle) and α -motor nerve terminals pathology, with the latter undergoing Wallerian-like degeneration (Diwan and Milburn 1986). Building on the functional studies described above that show the nerve is affected prior to skeletal muscle these studies suggest that α -motor nerve terminals may not only be vulnerable to hypoxia/ischaemia-reperfusion injuries but may also be the principal site of injury. The most conclusive study that indicates that α -motor nerve terminal may be principal site of injury in ischaemia-reperfusion injuries is that of Tombol *et al* (2002). These authors have shown progressive degeneration of rat α motor nerve terminals after 2hr tourniquet ischaemia followed by 2hr-5weeks reperfusion without any signs of ultrastructural changes to postsynaptic endplates or skeletal muscle fibres. The degenerative changes peaked between 24hr and 1week of reperfusion, with complete loss of synaptic vesicles, mitochondrial disruption, vacuolization and evidence to indicate activation of Schwann cells affecting 43% and 97% of nerve terminals innervating slow-twitch and fast-twitch muscles respectively. Signs of recovery were observed after a week but were not complete by the end of the experiment at 4 weeks with 26% and 37% of nerve

terminals from slow-twitch and fast-muscle muscles respectively still showing signs of pathological ultrastructure. In the most recent study, and the only study at light microscopy level, David *et al* (2007) have shown significant fragmentation and loss of α -motor nerve terminals from mSOD1 mice (see section 1.5.4) that express endogenous yellow fluorescent protein (YFP: see Chapter 4) in their neurons following 30min tourniquet ischemia and 6hr reperfusion. They also indicate that nerve terminals from wild-type animals that served as controls, are significantly less vulnerable than those from mSOD1 mice but their nerve terminals may also be lost following 30-60mins tourniquet application with reperfusion lasting somewhere between 6 and 24hrs (not specified). Important muscle fibre type differences were also highlighted, with α -motor nerve terminals from fast-twitch muscles being more vulnerable than those from slow-twitch muscles, consistent with the findings of Tombol *et al* (2002).

1.7.5 But why are these studies inconclusive and why are further studies needed?

Collectively, these studies do give some indication that α - nerve terminals are vulnerable to ischaemia reperfusion injury but are far from conclusive at demonstrating α -motor nerve terminal vulnerability, especially those that utilise experimental tourniquets (Makitie and Teravainen 1977a; Tombol *et al.* 2002; David *et al.* 2007). Tourniquets, by their very nature, cause significant compression to underlying tissues including peripheral nerves. In the clinical setting, tourniquets are applied with extreme care often by specially trained medical staff, with significant attention paid to tourniquet design, pressure, patients' resting blood pressure, local anatomy and limb diameter, and limbs are generally exsanguinated before application. This is done to ensure blood supply is reduced to clear the surgical field but not completely occluded to prevent oedema and to reduce necrosis, crush injuries, mechanical trauma and lesions to underlying muscle and nerves (e.g. Eckhoff 1931; McElvenny 1945; Fowler *et al.* 1972; Ochoa *et al.* 1972; Saunders *et al.* 1979; McEwen 1981; Nitz *et al.* 1986; McGraw and McEwen 1987; Pedowitz *et al.* 1992; Ohara *et al.* 1996; Kam *et al.* 2001; AORN 2007; Murphy and O'Connor 2007). This is because it is believed that the neurological complications following the use of tourniquets is caused primarily by mechanical stress and/or as a result of the physical lesion of nerves underlying tourniquets, particularly if tourniquets are applied incorrectly and over-pressurized (e.g., Eckhoff 1931; McElvenny 1945; Fowler *et al.* 1972; Ochoa *et al.* 1972; Rydevik and Nordborg 1980; Ohara *et al.* 1996; Kam *et al.* 2001; Kalla *et al.* 2003). While this does not rule out ischaemia-reperfusion injury at α -motor nerve terminals distal to the tourniquets, as discussed in section 1.5.1, crush injuries, lesions/ axotomy cause Wallerian degeneration of axons and nerve terminals with the first morphological signs *in vivo* being in α -motor nerve terminal within 12hr. Complete loss of distal region of α -motor neurons below the site of injury as a result of Wallerian degeneration probably accounts for many of the long-term neurological injuries seen in human patients. In experimental animal models of ischaemia-reperfusion injuries, tourniquets are not applied with the same care as

they are applied clinically. In particular, the two most recent studies describing structural α -motor nerve terminal pathology in response to ischaemia-reperfusion injuries (Tombol *et al.* 2002; David *et al.* 2007), have utilized elastic rubber bands wound around the hind limbs of rodents, a method that will significantly increase the risk of mechanical trauma to underlying nerves. This makes the degenerative changes identified in α -motor nerve terminals by Makitie and Teravainen 1977a, Tombol *et al.* 2002, David *et al.* 2007, and many of the other studies examining the effects of tourniquets, difficult to interpret. The data presented by David *et al.* (2007) does not conclusively show the loss of wild-type nerve terminals prior to 12hr in response to tourniquet ischaemia-reperfusion, i.e., before any structural changes caused by potential Wallerian degeneration induced by mechanical trauma to nerve. They do show that α -nerve terminals from mSOD1 are lost prior to the effects of Wallerian degeneration, but these nerve terminals are already known to be in a vulnerable, diseased state (see section 1.5.4). Of the studies that have shown structural pathology at α -motor nerve terminal following the use of tourniquets, only the study by Tombol *et al.* (2002) show some selective degenerative changes in α - nerve terminals before 12hr and prior to any potential influence of lesion induced Wallerian degeneration. The remaining studies utilise vascular occlusion/transection to induced ischaemia and therefore avoid the influence of lesion induced Wallerian degeneration but of these studies only one, Hatzipantelis *et al.* 2001, shows pathology at α -motor nerve terminals without any changes to muscle fibres. The remainder of studies therefore cannot rule out the possibility that structural changes observed in α -motor nerve terminals is not caused by postsynaptic/skeletal muscle pathology that is known to also cause loss of α -motor nerve terminals (Rich and Lichtman 1989; Bernstein and Lichtman 1999; McCann *et al.* 2007). Overall there has yet to be a study that conclusively demonstrates the functional and/or structural vulnerability of α -motor nerve terminals to ischaemia-reperfusion injury and therefore, assess their potential contribution to the neurological impairments seen following the application of tourniquets.

Further evidence for the potential involvement of α -motor neurons and their nerve terminals comes from a few isolated reports that *ex vivo* α -motor neurons are functionally vulnerable to hypoxia. By maintaining *ex vivo* rodent diaphragm muscles in hypoxic salines sparged⁶ with N₂ gas, studies have shown similar functional findings as the *in vivo* studies discussed above. Failure to elicit muscle fibre contraction via the phrenic nerve after 12-45mins hypoxia could be reversed with ~15min of reoxygenation (reperfusion) with O₂ gas or upon direct stimulation of the diaphragm (Krnjevic and Miledi 1959; Hubbard and Loyning 1966; Bazzi 1994; Zhu *et al.* 2006). Before the onset of neurotransmission failure, several authors have also observed massive increase in MEPP frequency (Hubbard and Loyning 1966; Nishimura 1986; Bukharaeva *et al.* 2005) followed by progressive decrease in EPPs and loss of contractile ability upon stimulation of the phrenic nerve (Hubbard and Loyning 1966). These studies therefore clearly indicate that in response to hypoxia there is a rapid increase in spontaneous vesicle release followed by progressive loss of the ability to evoke vesicle

⁶ Sparge meaning to introduce air or gas into a liquid (dictionary.reference.com)

release or conduction block. The mechanism(s) governing this response are still unknown but one study in rats has shown similar increases in MEPP frequency following metabolic inhibition with sodium azide (Anwyl and Ling 1983). Rabbit sciatic nerves removed and placed into mineral oil bubbled with N₂ gas show decreases in ATP and glucose within the first 2mins of hypoxia with levels becoming almost undetectable by 30-60min. This coincides with increases in lactate and AMP, which overall is consistent with inhibition of aerobic respiration, up-regulation of anaerobic respiration and metabolic fatigue (Stewart *et al.* 1965). These studies parallel the metabolic and functional effects of CNS hypoxic/ischaemic-reperfusion injuries where metabolic inhibition causes depolarisation of neurons that triggers massive vesicle release followed by conduction block. These functional events at nerve terminals are known to coincide rapidly with structural pathology that can lead to death of neighbouring neurons but also retrograde death of the parent neuron. What is still unclear in the PNS however is if α -motor nerve terminals are also structurally vulnerable to ischaemia/hypoxia-reperfusion injuries?

1.7.6 Summary and the present study

Functional and structural changes at nerve terminal have been identified to occur as principal events in a wide range of toxic, traumatic and degenerative neuropathologies where loss of synaptic connectivity leads to the manifestation of punitive symptoms (see Table 1 and section 1.5). The routine use of surgical and non-surgical tourniquets on peripheral limbs is also known to cause long-term neuropathies, but it is still unclear if pathology at α -motor nerve terminals because of hypoxia/ischaemia-reperfusion injuries are causing or contributing to the punitive complications of tourniquet use. A precedence for nerve terminal vulnerability has been already been set in CNS hypoxia/ischaemia-reperfusion injuries where functional and structural events at CNS nerve terminals are pivotal in mediating injury that leads to cell death, permanent disability and morbidity. The underlying reason(s) for this pivotal role is the susceptibility of nerve terminals to metabolic inhibition caused by the hypoxic element of ischaemia-reperfusion injury (e.g. Rothman 1983; Rothman 1984; Dewar *et al.* 1999; Lipton 1999; Ames 2000; White *et al.* 2000; Bickler and Donohoe 2002: see section 1.6) and to the fact that nerve terminals, in general, are known to have high energy demands (e.g. Wong-Riley 1989; Attwell and Laughlin 2001; Miller and Sheetz 2004; Hollenbeck and Saxton 2005), see section 1.4.1). This high energy demand combined with evidence indicating that the mitochondria contained within nerve terminals are more susceptible to inhibition, Ca²⁺ overloading and rupture than those in other neuronal compartments (John 1996; Davey *et al.* 1998; Brown *et al.* 2006; Naga *et al.* 2007) and may lead to the observed vulnerability of nerve terminals in a range of neuropathies, not just hypoxia/ischaemia-reperfusion injuries (Davey *et al.* 1998). In combination with other studies that indicate functional and structural changes, this gives good reason to believe that α -motor nerve terminals may also be vulnerable to hypoxia/ischaemia-reperfusion injuries. What can also be learnt from the CNS is that it is vitally important to have a range of different experimental model systems to investigate these injuries. Combinations of *in vivo*, *ex vivo* and *in vitro* experimental

models have been used not only to identify individual stimuli that contribute to hypoxia/ischaemia-reperfusion injuries, characterized by highly complex biochemical and cellular events, but also the underlying mechanisms by which they are governed. While a truly successful therapeutic strategy has yet to be developed to tackle CNS ischaemia-reperfusion injuries in human patients (Dewar *et al.* 1999; White *et al.* 2000; Brouns and De Deyn 2009), this method has provided a number of novel targets to direct therapeutic strategies against in the future (Dewar *et al.* 1999; Mehta *et al.* 2007; Brouns and De Deyn 2009). In the PNS, current studies are dominated by *in vivo* experimental model systems, either vascular occlusion or tourniquet application, which make identification of the major pathological stimuli caused by ischaemia-reperfusion injuries very difficult and further hinder identification of underlying mechanisms and the development of future preventative or therapeutic strategies. The major pathological stimuli that have been identified in other models systems, both in the CNS and in some *in vivo* PNS studies, are a lack of oxygen (hypoxia) followed by a period of reperfusion:

1.8 Aim

It is therefore my aim to determine the structural vulnerability of *ex vivo* adult mouse α -motor nerve terminals to hypoxia/ hypoxia-reperfusion injury.

Chapter 2

**Hypoxia-reperfusion injury causes rapid and selective disassembly
of α -motor nerve terminals.**

2.1 Introduction

Previous *ex vivo* studies of hypoxia-reperfusion injury have shown reversible functional changes at α -motor nerve terminals (Krnjevic and Miledi 1959; Hubbard and Loynning 1966; Anwyl and Ling 1983; Nishimura 1986; Bazzzy 1994; Zhu *et al.* 2006) but have not been used to show any structural changes that would indicate longer-term pathology. One possible reason for this may be the difficulty in generating physiologically relevant hypoxic environments in *ex vivo* model systems. There is no data on the normal *in vivo* O₂ levels (normoxia) at α -motor nerve terminals but the extracellular normoxic levels in the interstitial space of rodent skeletal muscle has been estimated to be between ~1 and 4% (Gorczyński and Duling 1978; Honig and Gayeski 1993; Shibata *et al.* 2001; Eu *et al.* 2003; Matsumoto *et al.* 2005), falling to as low as ~0.5% during muscle contractions (Gorczyński and Duling 1978; Klitzman *et al.* 1983; Eu *et al.* 2003). This low normoxic O₂ level is thought to be a mechanism that helps ensure O₂ supply from arterioles /capillaries to skeletal muscle by creating a steep O₂ gradient (Clanton 2007). It has been shown that the contraction force of mouse skeletal muscles is significantly improved when perfused with salines containing 1% O₂ compared to those with higher O₂ levels, indicating that this is a more physiological relevant O₂ level (Eu *et al.* 2003). α -motor nerve terminals are spatially related to the capillary network, for example, 12 μ m distant in the hamster cremaster muscle (Pierzga and Segal 1994) but do not appear to have overlying vasculature that would indicate they are not subject to significantly higher O₂ levels than skeletal muscles. Furthermore, it seems likely that α -motor nerve terminals may employ a similar adaptive mechanism in keeping low O₂ levels to help ensure O₂ supply as occurs in skeletal muscle. This low normoxic value (0.5-4%) in muscle fibres and their nerve terminals is an important issue that needs to be addressed in the experimental design of any *ex vivo* model systems that utilises environmental hypoxia rather than chemical inhibition of aerobic respiration, the former being a more physiological relevant stimulus. There is a general difficulty in reducing O₂ levels in physiological salines to below 0.8% due to atmospheric O₂ exchange and having to displace already dissolved O₂ (Clanton 2007). This value of 0.8% is well within the working range of skeletal muscle (0.5-4%) and presumably α -motor nerve terminals. Those studies that have shown function changes to α -motor nerve terminals in response to environmental hypoxia have recorded O₂ values ranging from 6-8% at or near the muscle surface (Bukharaeva *et al.* 2005; Zhu *et al.* 2006) while others have recorded an intramuscular value of 0-2.6% (Bazzzy 1994). This would indicate that in these experiments the level of O₂ might have varied from hypoxic to normoxic, and possibly even hyperoxic levels, with this degree of variance possibly further exacerbated across the depth of the muscle. As O₂ delivery relies on diffusion rather than supply from the vasculature, the muscle fibres/ α -motor nerve terminals located in the deeper regions are likely to be subject to much lower levels of O₂ (Eu *et al.* 2003; Zhu *et al.* 2006), particularly in the dense diaphragm muscle that these studies utilised. Overall, these studies and the model systems that they utilise may not be generating a truly hypoxic, physiologically relevant stimulus, with difficulty in interpretation of results further compounded by considerable degree of variability. By selecting small,

less dense skeletal muscles, reducing O₂ levels as low as possible and taking into consideration dissolved O₂ and atmospheric O₂ exchange it should be possible to reduce potential O₂ gradients within skeletal muscle/ α -motor nerve terminals, reduce variability and create more physiologically relevant O₂ levels in the *ex vivo* model system used in this study. In particular, if extracellular O₂ levels can be reduced below *in vivo* values of normoxia (0.5-4%) and near to *in vivo* values of hypoxia recorded in ischaemic limbs of surgical patients, 0.25% (Gustafsson *et al.* 1999; Matsumoto *et al.* 2005), it would be possible to create a more relevant hypoxic stimulus which, regardless of muscle fibre location (either superficial or in the central/deeper regions), would render the entire preparation hypoxic.

In previous studies, the Parson *et al* group have utilised *ex vivo* mouse lumbrical muscles found in the sole of the hind foot (see methods for details) which can be maintained under experimental conditions and used to examine structural response of α -motor nerve terminals using indirect fluorescence immunohistochemistry (Moores *et al.* 2005; Bettini *et al.* 2007). This neuromuscular preparation has been previously characterised, can be maintained in physiological saline (i.e., Krebs' solution) where variables may be controlled/monitored (e.g. O₂, pH and glucose) and the muscles are small allowing for better penetration of antibodies and O₂. This neuromuscular preparation also provides an interesting paradigm of the effects of lower limb tourniquet application and is composed of predominantly fast-twitch fibres (Betz *et al.* 1990; Murray *et al.* 2008a) that are more vulnerable to tourniquet induced ischaemia-reperfusion injury (Tombol *et al.* 2002; David *et al.* 2007). By necessity, in these preparations axotomy of the nerve supply occurs and over time (~12hr: see section 1.5.1) this will cause structural and functional loss of α -motor nerve terminals due to Wallerian degeneration. To prevent the complication of axotomy-induced α -motor nerve terminal pathology/loss, lumbrical muscles can be dissected with long distal nerve stumps extending up to the lower sciatic/upper tibial nerve (>2.5cm) to provide an additional 1-2hr per cm increase in the lag phase of Wallerian degeneration (Miledi and Slater 1970; Ribchester *et al.* 1995) where little change occurs in the distal axon and nerve terminal (Miledi and Slater 1968; 1970; Winlow and Usherwood 1976; Tsao *et al.* 1999; Gillingwater and Ribchester 2001). Wallerian degeneration is also temperature dependent, with a temperature of <25°C slowing the progression of Wallerian degeneration in isolated sciatic nerve from 2 to 7 days (Tsao *et al.* 1999). These measures have been previously shown to prevent functional and structural signs of Wallerian degeneration in the same neuromuscular preparations for at least 4hr (Bettini *et al.* 2007) and very similar preparations consisting of the *flexor digitorum brevis* for at least 6hr (Parson *et al.* 2004: see fig 2.1). With this in mind, I have elected that experimental time should not exceed 4hr with additional time to spare to complete dissection and pre-fixation processing. It is generally agreed that the upper limit for tourniquet application in human patients is 2hr (McElvenny 1945; Kessler 1966; Rorabeck 1980; Pedowitz *et al.* 1992; Kalla *et al.* 2003; Younger *et al.* 2005; Malanjam and Fischer 2006; AORN 2007; Kam 2007). As reoxygenation/reperfusion is key to recovery but also important in the injury process (e.g., Fowler *et al.* 1972; Makitie and Teravainen 1977b; a; Nitz *et al.* 1986; Zollman *et al.* 1991; Pedowitz *et al.*

1992; Iida *et al.* 2003; Wang *et al.* 2008), I have elected to use an experimental procedure consisting of 2hr hypoxia followed by 2hr reperfusion (reoxygenation): With this protocol any observed response(s) of α -motor nerve terminals can be largely attributed to hypoxia-reperfusion injury, rather than axotomy induced Wallerian degeneration. This is also the time frame shown by Tombol *et al.* (2002) to induce selective degenerative events in α -motor nerve terminals *in vivo* following tourniquet induced ischaemia-reperfusion injury.

Throughout the course of this study, I have used the term “reperfusion” to refer to the stage where O₂ is returned to the experiment. Use of either of the terms reoxygenation or reperfusion to refer to this stage can potentially be considered erroneous or confusing. “Reoxygenation” does not preclude in the first instance to the fact that glucose is present throughout the course of the experiment and that once the Krebs’ solution is reoxygenated contains all that is necessary for short to medium-term survival of neuromuscular preparation. Future studies (considered here but due to time restraints not carried out) may wish to investigate the potential role glucose has in both stages and would therefore need to be clearly distinguished from experiments that only examined the presence/absence of O₂ as the key variable. Alternatively, *in vivo* reperfusion injury involves as many complex co-stimuli as those of ischaemia (see section 1.6.1 and sections 1.7) which are not directly studied here (further discussed below in sections 2.4.2 and 2.4.3) hence use of the term “reperfusion” here may be considered erroneous. The important aspect of reperfusion injury however, is the ability of cells to reinstate metabolic activity (where both reinstatement and failure to do so contribute to most of the pathological stimuli of reperfusion injury) upon return of the blood supply. From a reductionist point of view (which was one of the aims of this study) it may be considered that the presence of glucose and O₂ are the two key aspects of reperfusion injury and it is for this reason why I have elected to use the term “reperfusion”, rather the term “reoxygenation” in this study.

In the following chapter, I describe the use of a novel experimental model system to show rapid and selective α -motor nerve terminal pathology in response to 2hr hypoxia, 2hr reperfusion. The resulting α -motor nerve terminal pathology is indicative of on-going nerve terminals disassembly with evidence to indicate that multiple mechanisms of α -motor nerve terminal loss may be active.

2.2 Methods

2.2.1 Animals and skeletal muscle dissection

Tissue was obtained from female 8-12 week old C57Bl/6 mice (Harlan-Olac, UK). Only female mice were used to avoid possible gender differences that have been highlighted in ischaemic brain injury (Alkayed *et al.* 1998; Bramlett 2005) and female mice are smaller (Gall and Kyle 1968) with less fatty tissue deposits (Parson *et al.*: unpublished observations) that helps improve antibody penetration, quality of immunohistochemistry and O₂ diffusion. Mice were culled by a rising CO₂ concentration or cervical dislocation in accordance with the Animal (Scientific Procedures) Act 1986 (no difference was noted in the experimental data with these two culling methods). Tissues were dissected under a Zeiss binocular microscope in silicone-lined (Sylgard 184: Dow Corning, Germany) petri dishes. The tissues were maintained in Krebs' solution (144 mM Na⁺, 5 mM K⁺, 2 mM Ca²⁺, 1 mM Mg²⁺, 131.2 mM Cl⁻, 23.8 mM HCO₃⁻, 0.4 mM H₂PO₄²⁻, 5 mM D-glucose, 5.5mM HEPES⁷: pH 7.2-7.4) sparged, via a sintered glass bubbler, with 95%:5% O₂:CO₂ gas. Extreme care was taken in all instances to ensure that each preparation was free of dissection damage and with as much of the overlying tissues removed as was practically possible. In this study, α -motor nerve terminals were studied from 4 deep *lumbrical* muscles, which are composed of predominantly fast-twitch fibre types (Betz *et al.* 1990; Murray *et al.* 2008a). These four small muscles (~2.5mm in length, ~1mm in diameter) are found in the second layer of the plantar aspect of the foot. They are numbered 1 to 4 according to their anatomical location from the *hallux* (big toe: see fig 2.1) and are involved in flexing distal and proximal *phalanges* of the lateral four digits. The hind limbs of the mice were amputated at the level of the thigh, skinned and pinned out in petri dishes. The sciatic/tibial nerve protruding from the thigh was carefully released from the surrounding tissues and tendons as it tracked through the lower hind limb until it reached the level of the *calcaneus* (heel bone). The *plantaris* and *flexor digitorum brevis longus tendon* was then severed at the attachment point on the *calcaneus*, avoiding the sciatic/tibial nerve stump sandwiched between the two tendons as it continues into the sole of the foot. The 4 deep lumbrical muscles attached to the *plantaris* tendon were dissected free from the foot and midway up the digits along with the overlying *flexor digitorum brevis* muscles and *flexor digitorum brevis longus tendon*. The *flexor digitorum brevis* muscle and *flexor digitorum brevis longus tendon* remained in place until after experimentation and fixation to ensure the nerve supply to the *lumbrical* muscles was not damaged. To ensure that *lumbrical* muscle preparations from the left and right hind foot could be identified and that each of the four *lumbrical* muscles could be correctly assigned a number, a small suture was tied in the *plantaris* tendon of the right hind leg. As a long nerve stump, >2.5cm in length, is particularly vulnerable to dissection damage, especially around the heel bone/ankle joint I verified their integrity before use in experiments by stimulating the nerve stump with a suction electrode (custom made) connected to an isolated stimulation and trigger unit (Model DS2A, Digitimer Ltd,

⁷ 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid: Sigma

Plantar aspect of the human foot

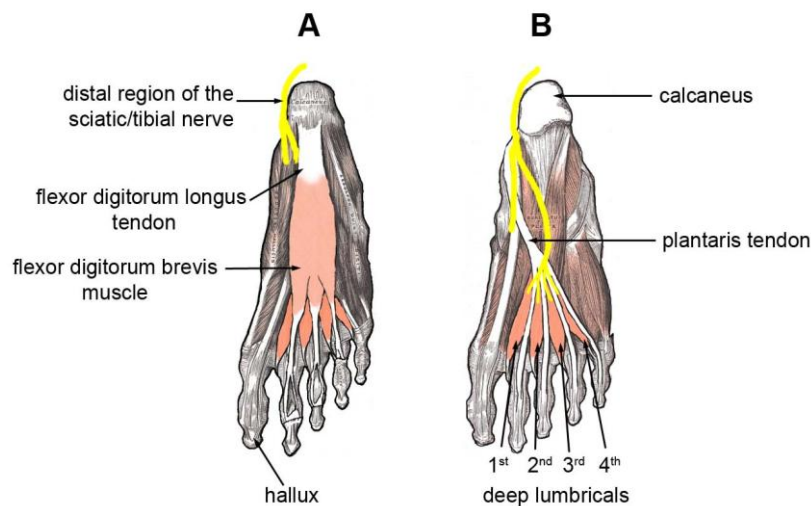


Fig 2.1: The plantar aspect of the human foot showing the lumbrical muscles that, except for relative proportions, are very similar to that of the mouse. The pseudo-colour tissues of the first and second layer of the sole of foot shown in (A) was removed from the rest of the foot at the beginning of the experiment but with the sciatic/tibial distal nerve stump extending up to level of the mid-thigh. Following 2H-2R or control procedures and fixation, the first layer of the sole of the foot was removed (flexor digitorum longus tendon, flexor digitorum brevis muscle and distal nerve stump) to fully expose the 4 deep lumbrical muscles attached to the plantaris tendon (B). This was to ensure that the nerve supply to the deep lumbrical muscles, which is sandwiched between flexor digitorum longus and plantaris tendons, was not damaged prior to the start of the experiment. Careful note of the position of the hallux ('big toe') was taken so the each of the deep lumbrical could be assigned its correct anatomical number (1-4). Image adapted from the bartleby.com edition of Gray's *Anatomy of the Human Body*.

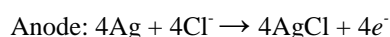
Hertfordshire, UK). Where tested, all muscles produced a strong twitch response with a 0.2ms pulse at 0.5V or below. From time of death to the start of the experiment typically took no more than 1hr. To help with visualisation of motor nerve terminals from lumbrical muscles and after processing for immunohistochemistry was complete, each of the four lumbrical muscles was isolated from the *plantaris*, had overlying connective tissues removed and were mounted onto their lateral surface.

2.2.2 Hypoxia-reperfusion model system

250ml HEPES buffered Krebs' solution was vigorously bubbled with 95%:5% N₂:CO₂ via a sintered glass bubbler for a minimum of 1.5hr in a 250ml conical flask prior to the start of experimentation. The long setup period was required to ensure maximum displacement of already dissolved O₂ and the use of a conical flask created a high N₂, low O₂ micro-environment coupled with a small surface to reduce atmospheric oxygen exchange (see Fig 2.2). Muscles dissected from the right-hand side, with their respective nerve stumps, were subject to 2hr of hypoxia followed by 2hr reperfusion in 95%:5% O₂:CO₂ sparged Krebs' solution. Muscles from the left-hand side of the same animals were maintained as experimental control specimens in 95%:5% O₂:CO₂ sparged Krebs' solution also in a

conical flask for the duration of the experiment (4hr). All muscles were left free floating with sufficient flux so as not to become stagnant but to remain below the surface during the course of the experiment.

Oxygen: The system was monitored with a Clark-type (Clark *et al.* 1953) micro-oxygen electrode (MLT1120, AD Instruments, UK/ MI-730 Microelectrodes Inc, USA). The electrode, composed of a platinum cathode and a silver/silver chloride anode, is enclosed in a housing chamber filled with electrolyte solution. The tip of the housing chamber (3mm) is covered with a Teflon membrane that allows passage of O₂ into the supplied electrolyte solution. When the platinum cathode is held at 0.8V, the following redox reaction occurs to produce a current proportional to the concentration of the O₂ diffusing across the membrane.



The O₂ electrode was connected to an analogue adapter unit (MLT1122) and 8/30 Powerlab bridging amp (ML870: both AD Instruments, UK) with data recorded on an Apple Mac G3 computer and MacLab/Chart 4 software (AD Instruments, UK). Prior to the start of each experiment, the electrolyte solution was replaced and the electrode underwent two-point calibration in ~40ml of distilled water placed on a magnetic stirring platform. Both solutions were left to equilibrate for at least 10mins, one with ambient air that was later used to calibrate the electrode to 21% O₂. The other solution was rendered anoxic (0% O₂) with the addition of sodium sulphite (~2g) before being left to equilibrate and the addition of a further ~2g of sodium sulphite at the point of calibration. During the course of the experiment, the electrode was placed well below surface level and away from the line of bubbles produced by sparging. O₂ levels were recorded every 10s over a 10V range, with the signal filtered for mains noise.

Temperature: Temperature was monitored via a thermistor probe (2mm tip) and a temperature controller unit (BSC-T3 and TC-202A respectively, Harvard Apparatus, Hertfordshire, UK). The controller unit was connected to the Powerlab bridging amp and Mac computer as above and set to record temperature continuously with a scaling factor of 100mV per °C as indicated by manufactures instructions and confirmed via the temperature controller's display.

pH: pH was monitored visually using an analogue volt meter (Corning Eel model 7 pH meter) with a combination pH electrode (Thermo Russell) that was calibrated to pH7 at the start of each experiment. pH was maintained in the Krebs' solution by using bicarbonate/CO₂ and HEPES buffer

2.2.3 Fluorescent Immunohistochemistry

At the end of the experiment, post-synaptic acetylcholine receptors were labelled by incubation in tetramethylrhodamine isothiocyanate-conjugated α -bungarotoxin (BTX) for 7mins (2.5 μ g/ml in oxygenated Krebs' solution: Invitrogen) prior to fixation in absolute methanol (MeOH) at -20°C for 15mins. Tissues were washed in phosphate buffered saline (PBS, Sigma) and immersed in a blocking

solution (0.1% TX-100, 0.2% IgG/protease free bovine serum albumin (Jackson ImmunoResearch, Stratech, Newmarket, UK), 0.1% sodium azide in PBS) for a minimum of 1.5hr. This was followed by at least 16hr incubation on a rocking platform with monoclonal anti-neurofilament (NF) 165kDa primary antibodies (1:250: Developmental Studies Hybridoma Bank, Iowa, USA) diluted in blocking solution at 4°C. This was followed by 3x10min washes in PBS and a subsequent 1hr incubation in Cyanine2 (Cy2) donkey anti-mouse secondary antibodies (1:500; Jackson ImmunoResearch) diluted in blocking solution. Following 3x20min wash in PBS, the above process was repeated but with anti-synaptic vesicle protein 2 (SV₂) primary antibodies (1:250: Developmental Studies Hybridoma Bank). Following the final wash for 2x10min and 2x15min in PBS muscles were mounted on to glass slides in 4% n-propylgallate in glycerol and stored in the dark at 4°C.

Alternatively and as indicated in the text, tissues were directly fixed by immersion in freshly made PLP⁸ fixative (4% liquid formaldehyde (Electron Microscopy Sciences, PA 19440, USA), 1.8% lysine, 0.2% sodium-m-periodate: McLean and Nakane 1974) for 30mins before washing for a minimum of 3x10min in PBS containing 0.5% TX-100. After incubation in blocking solution for 1hr, the above sequential staining protocol was used but with the following combinations of primary antibodies: rabbit anti- β -tubulin III (1:100: Abcam), goat anti-Choline Acetyltransferase (1:100: Calbiochem) and SV₂ (as above). These antibodies were visualised with appropriate Cy2 or Cyanine 3 (Cy3) secondary anti-rabbit, goat, or mouse antibodies (1:500; Jackson ImmunoResearch) respectively. Muscles were then washed 2x10min and incubated in alexa647-conjugated α -bungarotoxin at 5 μ g/ml in PBS for 30min. Following a final wash in PBS muscles were mounted and stored as above.

2.2.4 Electron Microscopy

At the end of some experiments, lumbrical muscles were fixed in a 2.5% Glutaraldehyde/4% formaldehyde solution in 0.1M phosphate buffer for 6hr. The 2nd lumbrical muscle was isolated and washed 3x10mins in 0.1M phosphate buffer (PB). Muscles were then post-fixed in 1% osmium tetroxide (in 0.1% phosphate buffer) for 45mins, followed by 3x10min wash in PB and dehydration through an ascending series of ethanol solutions (30-100% ethanol). Following immersion in propylene oxide for 2x10mins, muscles were embedded in Durcupan resin and left to set for 24-48hr at 60°C. 70-90nm sections were cut on an ultramicrotome, floated onto Formvar-coated grids (Agar Scientific, UK) and impregnated with heavy metal stains (uranyl acetate and lead citrate) in a LKB 'Ultrastainer'. Sections were viewed on a Philips CM12 transmission electron microscope and negatives were scanned on to PC using an Epson (Long Beach, CA) 4870 photo flatbed scanner

⁸ PLP is the acronym for this fixative developed by McLean and Nakane (1974) standing for Paraformaldehyde-Lysine-Periodate. It is a common mistake that formaldehyde is often referred to paraformaldehyde: the latter refers to the solidified polymerised form (*para* meaning polymer) while the former refers to the liquid monomeric form. To avoid confusion I will use the PLP acronym used by developing authors (and many others) throughout the course of this study.

equipped with transparency adaptor at 800dpi. Minor adjustments for brightness and contrast were made with Adobe Photoshop CS2 software.

2.2.5 Image Capture, Quantification and Statistical analysis

All fluorescent immunohistochemical preparations were examined to ensure quality of immunohistochemistry. Preparations were then quantified on an epifluorescence upright microscope (Zeiss Standard 10) fitted with a FITC/TRITC filter set and 50W mercury vapour light source. Fluorescence images were captured using x25, 0.6NA or x50, 0.8NA water immersion objectives (Zeiss) via a monochrome Cohu 4910 CCD camera (Vista Vision, Stevenage, UK) and frames averaged (2-100 frames) using Scion Image capture software (Alrad, UK) running on an Apple Macintosh Quadra 650 microcomputer. Image capture was also carried out on BioRad Radiance 2000 laser scanning confocal microscope (x40, 0.8NA oil-immersion objective) using Lasersharp 2000 software on PC to project composite images of serial optical sections. All images had minor adjustments for brightness and contrast and were coloured and reconstructed as required using Adobe Photoshop CS2 software on PC.

All quantification of NF/SV₂ labelled muscles was carried out at x25 magnification. Muscles were assessed to ensure that there was no significant muscle fibre death beyond one or two dead muscle fibres seen at the edge of the muscle preparation (assumed to be the result of minor dissection damage). The postsynaptic endplates were then quantified from top right to bottom left through the depth of the muscle, with every attempt made to ensure the whole population was counted. There were however, the following exclusions: endplates that were right angles to the plane of view, obscured from the plane of view (e.g. by intramuscular nerve or debris) or belonged to a muscle fibre that lacked striations or had significant autofluorescence. The α -motor nerve terminals opposing each endplate were assessed and categorised as follows:

Full: NF/SV₂ immunoreactivity fully opposed postsynaptic BTX staining of the endplate.

Partial: NF/SV₂ immunoreactivity did not fully oppose postsynaptic BTX staining of the endplate. To account for normal ongoing synaptic re-organisation (synaptic guttering) endplates were only classified as being partially occupied when NF/SV₂ immunoreactivity was absent from a region that corresponded to approximately two or more synaptic boutons.

Vacant: No clearly distinguishable NF/SV₂ immunoreactivity could be detected opposing the muscle endplate.

All numerical data was collated using Microsoft Excel 2003 on PC. The percentage of full, partial and vacant endplates were calculated per lumbrical and then pooled. All values are percentage mean \pm standard error of the mean (SEM), where N = the number of animals, n =the number of muscles with

total number of endplates counted enclosed in brackets. Statistical testing was carried using GraphPad Prism 4 software. Multiple testing was carried out using a Kruskal-Wallis test, the non-parametric equivalent of an ANOVA (Dytham 2003), followed by a Dunn's post-hoc test to identify which group of pairs were statistically different. Those pairs identified as being statistically different underwent further testing with a pairwise Mann-Whitney U-Test, a more powerful and less conservative test, and resulting *P*-values reported. Only selecting pairs already identified as being significant in the Kruskal-Wallis /Dunn test avoided having to reduce the critical threshold of *P* to below 0.05 to compensate for cumulative errors (Dytham 2003).

2.3 Results

2.3.1 Characterisation of the experimental system designed to model hypoxia-reperfusion injury.

To successfully determine the vulnerability of α -motor nerve terminals to hypoxia-reperfusion injury it was important to generate a model system in which *ex vivo* neuromuscular preparations could be monitored and maintained in a constant, physiologically relevant hypoxic environment. *In vivo*, the normoxic working range of rodent skeletal muscle has been estimated to be 0.5-4% (Gorczynski and Duling 1978; Klitzman *et al.* 1983; Honig and Gayeski 1993; Shibata *et al.* 2001; Eu *et al.* 2003; Matsumoto *et al.* 2005) but due to atmospheric O₂ exchange and already dissolved O₂, there is considerable difficulty in reducing O₂ to below 0.8%, (Clanton 2007). To help overcome this difficulty, preparations were maintained in a conical flask with 250ml of HEPES buffered Krebs' solution. By using the conical flask and vigorously sparging with 95%:5% N₂:CO₂ gas, it was aimed to reduce the surface area to volume ratio, increase the displacement of already dissolved O₂ and create a low O₂ micro-environment at surface level to reduce atmospheric oxygen exchange (Fig 2.2A). Monitoring this system with a calibrated O₂ electrode showed that O₂ levels typically fell below 0.5% within 15-30mins after sparging commenced (Fig 2.2B) but O₂ levels could continue to fall for a further 45mins (1.25hr from the start) until a steady state level was reached at between 0.1% and 0.25%. This steady state level of <0.25% is in agreement with previously reported O₂ values *in vivo* following tourniquet-induced ischaemia (Gustafsson *et al.* 1999; Matsumoto *et al.* 2005). Due to this delay in producing a steady-state O₂ level, all subsequent experiments were allowed to equilibrate for a minimum of 1.5hr before being used to induce hypoxia in *ex vivo* nerve/muscle preparations, thereby improving the consistency of the hypoxic environment. Following the set-up (1.5hr) and hypoxic (2hr) phases of the experiment, a 2hr phase of reperfusion was carried out by introducing 95%:5% O₂:CO₂ gas that, within minutes, produced a dissolved oxygen concentration in the region of 54-59%. Control experiments were maintained for 4hr in a conical flask with 250ml of HEPES buffered Krebs' solution sparged with 95%:5% O₂:CO₂. Monitoring the system with a pH electrode showed that no significant changes in pH occurred during the course of the experiments. Temperature was also monitored to show that this remained relatively consistent within the same experiment and between experiments (22 - 24.5°C).

2.3.2 Neuromuscular preparations and control morphology

To assess potential α -motor nerve terminal pathology following 2hr hypoxia - 2hr reperfusion (2H-2R) a fluorescent immunohistochemical staining protocol targeted at synaptic vesicle 2 protein (SV₂) and neurofilament 168kDa (NF) was modified from those previously established by Parson *et al.*

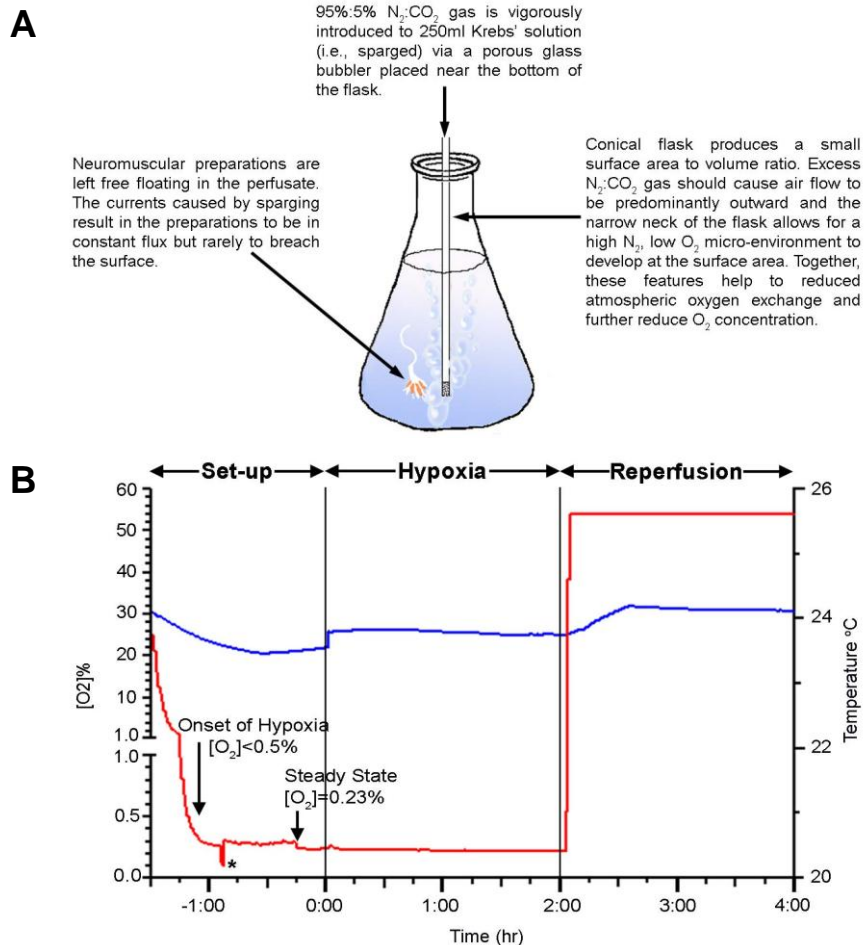


Fig 2.2 Hypoxia-reperfusion model system. (A): Schematic representation of the hypoxia-reperfusion model system with key principals/ assumptions indicated. Monitoring of the system took place by positioning oxygen, temperature and pH probes well below surface level and away from the direct line of the bubbles (not shown). (B): Example recording from the model system indicated in (A) showing O_2 concentration (red line) during the system set-up phase and the experimental phases of hypoxia and reperfusion. In the set-up phase, 250ml of Krebs' solution is sparged with 95:5% N_2 : CO_2 gas and O_2 concentration is seen to reach below the lowest reported threshold for skeletal muscle normoxia, i.e., less than 0.5%, within 30mins. In the recording shown, hypoxic conditions begin after 19mins (-1.2hr). The O_2 concentration however, could continue to fall for a further 1hr until it reached a consistent, steady state. In all experiments where recordings took place, steady O_2 state was below 0.25% and in this example recording reached the mean value of 0.23% after 1hr 14min (-16mins). To ensure that oxygen levels remained as consistent as possible, all hypoxia-reperfusion experiments began after the Krebs' solution was sparged with 95:5% N_2 : CO_2 for 1.5hr (system set-up phase). After the set-up phase, skeletal muscle preparations were added to the Krebs' solution to begin the hypoxic phase which lasted for 2hr. This was followed by 2hr reperfusion using 95:5% O_2 : CO_2 gas. In the recording above, O_2 concentration exceeded 0.5% within 2mins and was maximal by 5mins (54%) which were typical values for all experiments. After reperfusion, preparations were removed and processed for immunofluorescent staining. Temperature was monitored throughout and was maintained at 23-24 $^{\circ}C$ (blue line). pH was also monitored throughout experiments and maintained between 7.2-7.4 (not shown). Control muscles were maintained in 250ml Krebs' solution sparged only with 95:5% O_2 : CO_2 gas for the full 4hrs duration. (Asterisk indicates recording artefact.)

(2004) and Bettini *et al.* (2007). This included changing the fixative from formaldehyde to MeOH⁹, use of IgG/ protease free bovine serum albumin (that can help in the reducing non-specific binding of antibodies (Jackson ImmunoResearch/Strattech product information)), sequential application of antibodies and increasing the length of time tissues were in the blocking and primary antibody incubation stages (factors influencing antibody binding and immunohistochemistry (and in particular fixation) are further discussed in Chapter 5, section 5.4.1 and 5.4.2). The result of these changes produced NF/SV₂ immunoreactivity that was robust and intense across the length and depth of individual lumbrical muscles (Fig 2.3) and provided the consistency required to establish the parameters for control morphology (see Fig 2.7 for quantification of control morphology) and identification of pathological morphology. By comparing presynaptic NF/SV₂ immunoreactivity with that of post-synaptic acetylcholine receptors (endplates) identified with α -bungarotoxin (BTX) showed that under control conditions, NF/SV₂ immunoreactivity completely and fully opposed that of the BTX in >95% of cases. A small population of endplates were seen to have no NF/SV₂ immunoreactivity in a small part of their overlying nerve terminal and in most cases appeared to correspond with approximately one nerve terminal bouton. This is thought to represent on-going synaptic plasticity at nerve terminals and has been previously been termed ‘synaptic guttering’ (Cardasis and Padykula 1981; Wernig and Herrera 1986; Wigston 1989). Due to this, α -motor nerve terminals were classified as having a normal, ‘full’ morphology as long as NF/SV₂ was absent from no more than one terminal bouton. Rarely (<~2%), endplates were identified with no NF/SV₂ immunoreactivity in two or more boutons and were therefore categorised as having a ‘partial’ α -motor nerve terminal morphology. Occasionally, some endplates in control muscles (<~2%) were also identified with no opposing NF/SV₂ immunoreactivity and categorised as ‘vacant’. Like synaptic guttering, this is probably the result of on-going synaptic plasticity and/or the result of a naturally occurring injury (examples of partial and vacant α -motor nerve terminal morphologies in control preparations can be seen in Chapter 3, Fig 3.6 and Fig 3.10 respectively)

2.3.3 Post-synaptic endplate and muscle fibre morphology is maintained following hypoxia-reperfusion injury

The effects of 2hr hypoxia followed by 2hr reperfusion (2H-2R) on the morphology of pre- and post-synaptic components of the mouse neuromuscular junction in the 4 deep lumbrical muscles from the right hind leg ($N=7$, $n=28(4974)$) were compared with those muscles from the left hind leg kept under control conditions ($N=7$, $n=28(5090)$). 2H-2R did not cause any identifiable changes to the integrity/morphology of post-synaptic endplates or muscle fibres compared with control preparations

⁹ The anti-NF 165KDa antibody has been used with formaldehyde fixation for a number of years by the Parson *et al.* group providing intense and robust staining. It was noted independently by the Parson *et al.* and Gillingwater *et al.* groups (personal communication from Dr. Simon Parson and Dr. Tom Gillingwater, University of Edinburgh) that, despite no change to processing protocols, there had been a progressive loss of immunoreactivity with several different batches/lots of this antibody. This surprising (the antibody is both monoclonal and from a hydridoma cell line) loss of immunoreactivity with established protocols was the reason why old protocols had to be adapted to include changes in fixation and incubations times etc., (see above).

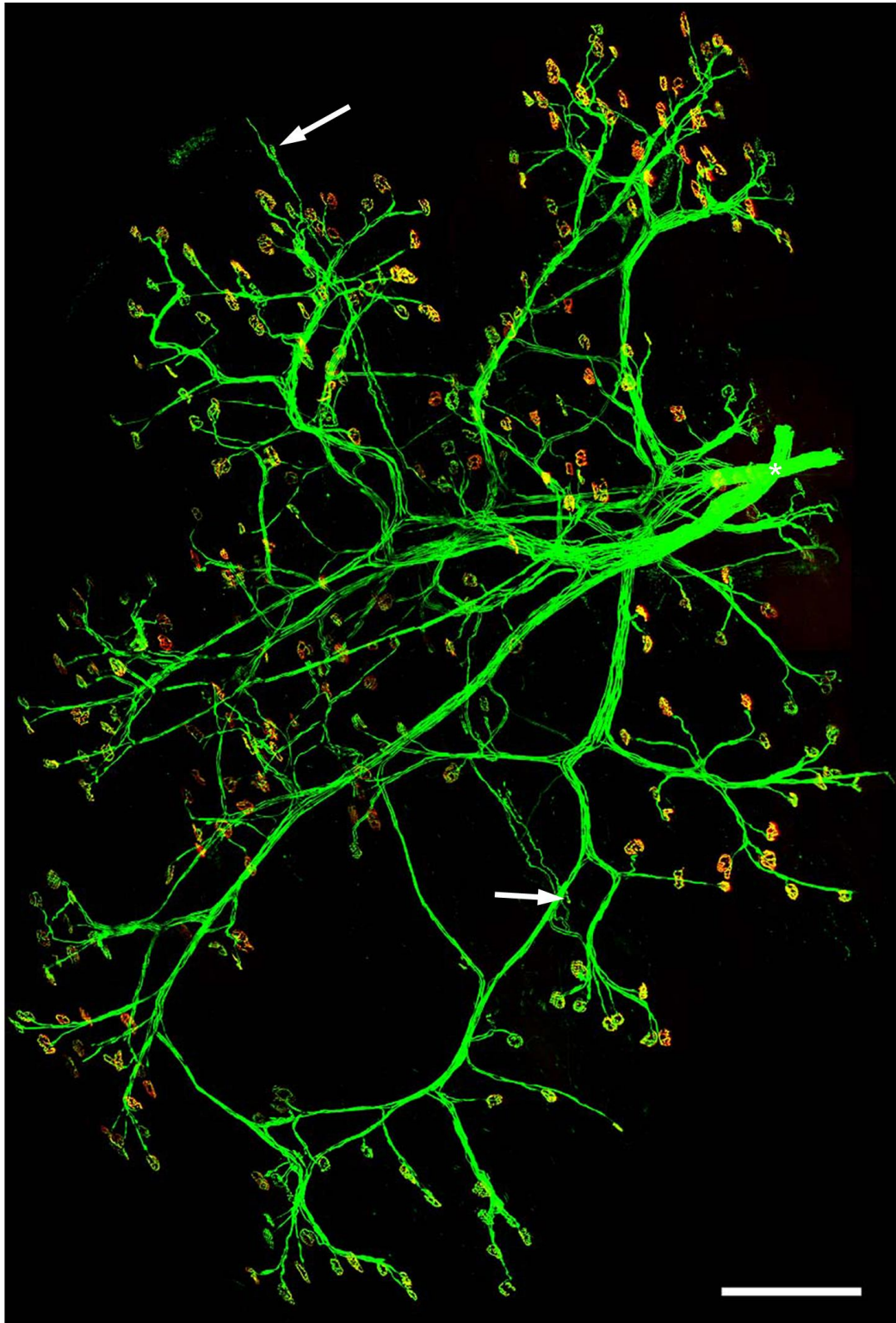


Fig 2.3: Reconstruction of a lumbrical muscle kept under control conditions.

Reconstructed confocal projections of a mouse lumbrical muscle (8-12wks) showing there is no significant α -motor nerve terminal pathology after being maintained in control conditions (4hr in 95%:5% O₂:CO₂ sparged Krebs' solution). Presynaptic neurofilament 165kDa (NF) and synaptic vesicle 2 protein (SV₂) immunoreactivity (visualised with Cy-2 secondary antibodies: GREEN) can be seen to fully oppose post-synaptic endplates labelled with TRITC-conjugated BTX (RED). The main branches of the intramuscular nerve (*) can be seen to send a complex network of collateral branches through the length and depth of the muscle. These collateral branches end in nerve terminal arborisations and oppose post-synaptic endplates located on individual muscle fibres. γ -neuromuscular junctions are present (arrow: see chapter 3), although no IA/II nerve endings can be identified. Immunoreactivity in general, appears robust and relatively intense allowing for consistent morphological analysis. Scale bar 200 μ m.

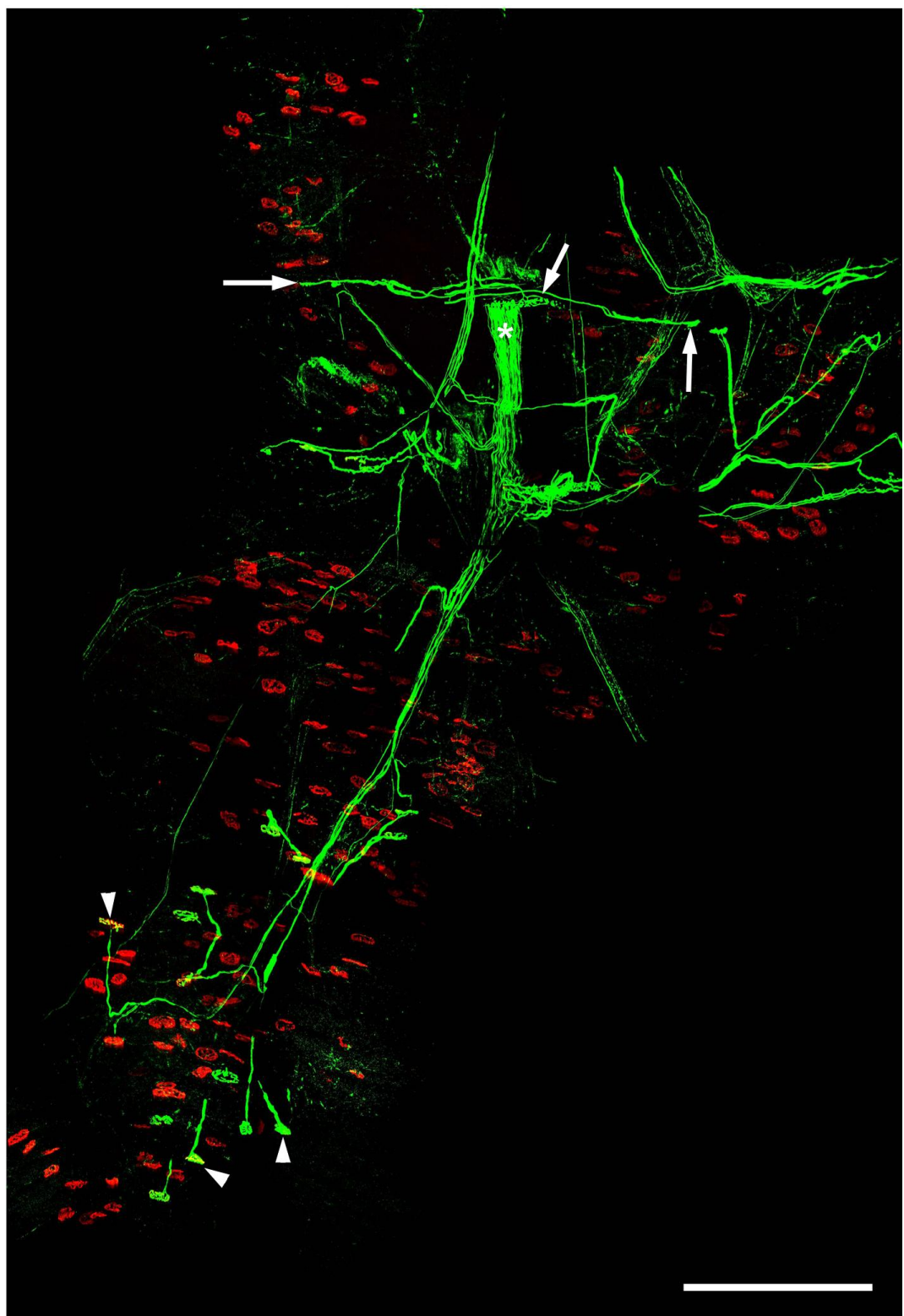


Fig 2.4: Reconstruction of a lumbrical muscle following 2hr hypoxia-2hr reperfusion (2H-2R). Reconstructed confocal projections of a mouse lumbrical muscle (8-12wks) showing widespread loss of presynaptic neurofilament 165kDa and synaptic vesicle 2 protein (NF/SV₂) immunoreactivity (visualised with Cy-2 secondary antibodies: GREEN) leaving most post-synaptic endplates labelled with TRITC-conjugated BTX (RED) appearing vacant. The main intramuscular nerve (*) can still be identified but the complex network of axon collaterals is reduced and often discontinuous. Some endplates however, still remain with some opposing NF/SV₂ immunoreactivity in their presynaptic α -motor nerve terminals and pre-terminal axons (e.g., arrowheads). γ -motor nerve terminals and IA/II sensory endings can still be identified (arrows: see section Chapter 3). Scale bar 200 μ m.

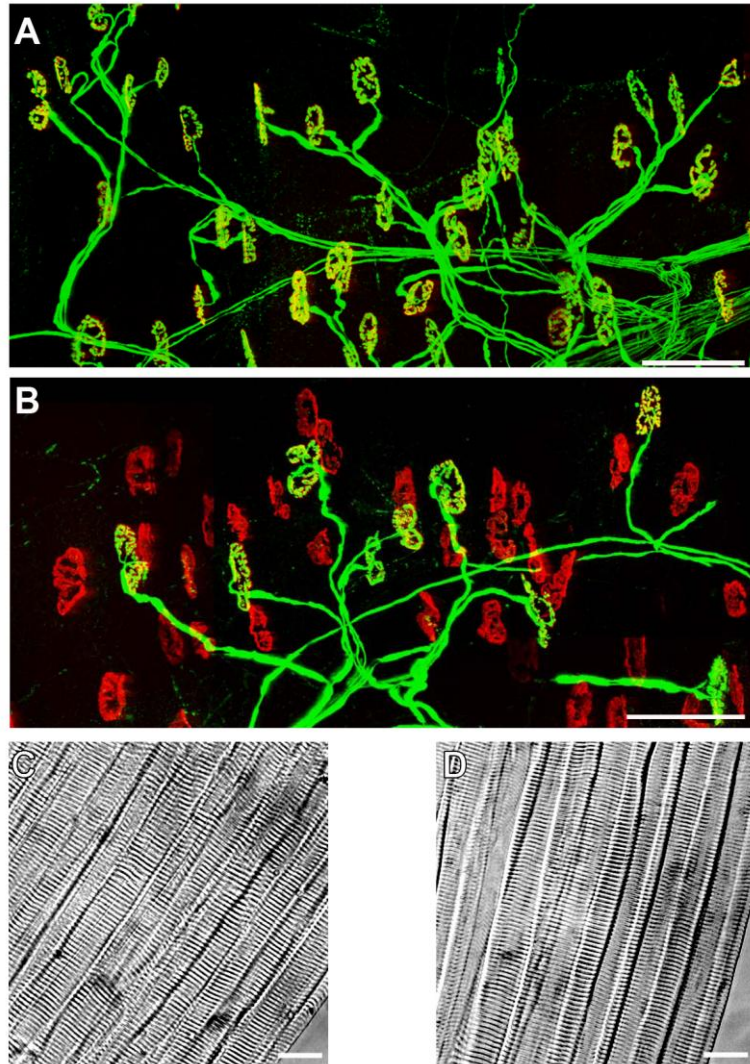


Fig 2.5: Selective pathology in α -motor nerve terminals following hypoxia-reperfusion injury. In control conditions (**A**) confocal projections demonstrate the characteristic 'pretzel-like' morphology of postsynaptic endplates (visualised with BTX: RED) which are fully opposed by an overlying nerve terminal arborisation, identifiable by NF/SV₂ immunoreactivity (GREEN). Axons can also be clearly traced back into the nearest collateral branch and phase-contrast micrographs (**C**) show uniform muscle fibres with clear striations. Following 2H-2R, confocal projections (**B**) show that postsynaptic endplates appear unchanged but a large proportion of endplates have lost all opposing NF/SV₂ immunoreactivity in their presynaptic nerve terminals and pre-terminal axons. Muscle fibres (**D**) appear unaffected by 2H-2R with a uniform structure and clear striations visible in phase-contrast micrographs. Scale bars 10 μ m (A&B), 50 μ m (D&C).

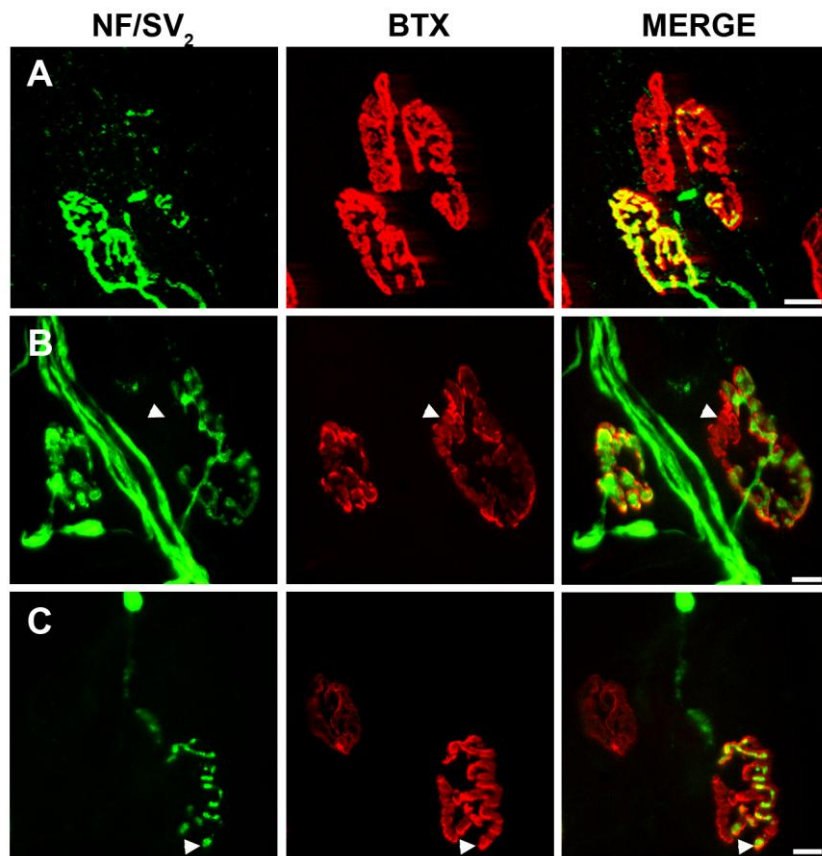


Fig 2.6: A small population of α -motor nerve terminals show Wallerian-like and withdrawal-like mechanisms of disassembly. Endplates that are only partially occupied or in the intermediate stage of breakdown can provide morphological insights into the mechanisms of α -motor nerve terminal pathology following hypoxia-reperfusion injury. **(A)** Confocal micrographs showing immunohistochemically labelled neuromuscular junctions in lumbrical muscles following 2H-2R treatment. One endplate (RED) remained fully occupied (bottom left) with 'full' overlying NF/SV₂ immunoreactivity (GREEN). A neighbouring endplate (upper middle; less than 50 μ m away) had no residual NF/SV₂ immunoreactivity (vacant). A third endplate (upper right) was partially occupied by overlying NF/SV₂ immunoreactivity. However, note how the presynaptic staining is fragmented in both the motor nerve terminal and pre-terminal axon. This would be synonymous with nerve terminal loss by a Wallerian degeneration-like process. **(B)** Micrograph showing another example of a partially occupied endplate (arrowhead) which remains entirely connected to its incoming pre-terminal axon (i.e. no fragmentation). The endplate has lost approximately 25% of its overlying nerve terminal while the remainder appears normal. This type of partial loss is indicative of nerve terminal loss by an asynchronous, withdrawal-like process. **(C)** Micrograph showing another example of a partially-occupied endplate with complete loss of NF/SV₂ immunoreactivity in one part of the nerve terminal, much like the asynchronous, withdrawal-like process observed in (C). This was also accompanied by the presence of isolated nerve terminal fragments (arrowhead) like that seen in (A) which is opposed by the endplate. Note however, that the pre-terminal axon presents with weak NF/SV₂ immunoreactivity, compared to the fragmented or normal immunoreactivity seen in (A) and (B) respectively. Scale bar 10 μ m.

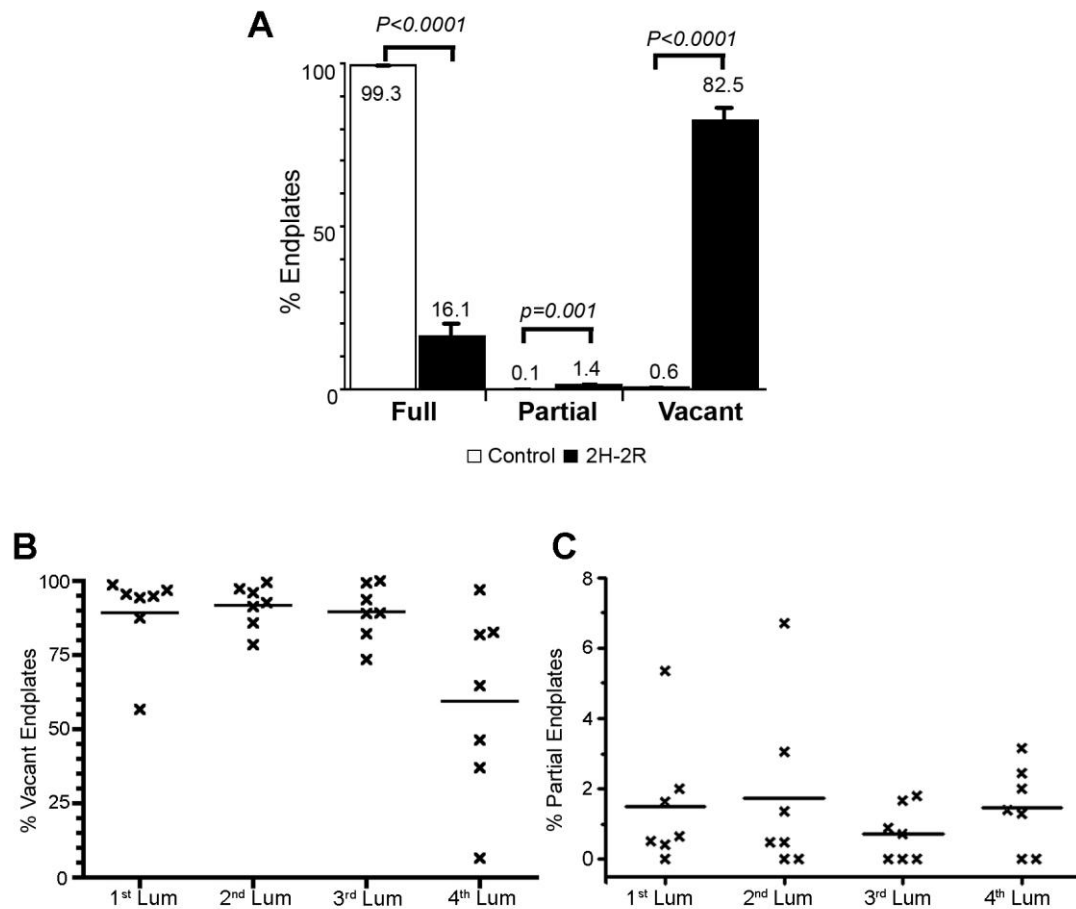


Fig 2.7 Quantification of α -motor nerve terminal response to hypoxia-reperfusion injury compared with those maintained in control conditions. (A), Bar chart (%mean \pm SE) showing the categorical quantification of α -motor nerve terminal status (i.e., fully occupied, partially occupied or vacant) that oppose post-synaptic endplates from hypoxia-reperfusion treated (2H-2R: black bars) and control (white bars) lumbrical muscles. Results were statistically analyzed (see methods) to show that there was a highly significant increase in both vacant and partial morphologies and a concurrent decrease in full nerve terminal morphologies following 2H-2R injury compared with controls. **(B),** Scatter plot (%mean indicated by line) showing intra-lumbrical differences in the number of endplates identified with no NF/SV₂ immunoreactivity, i.e., vacant, following 2H-2R injury. Statistical analysis showed there is no significant difference between the 4 lumbricals however, it is clear that the 4th lumbrical has a highly variable response to 2H-2R. **(C):** Scatter plot (%mean indicated by line) showing intra-lumbrical differences in the number of endplates identified with partial nerve terminal morphology following 2H-2R injury. There is no statistical difference in the number of partially occupied endplates between lumbricals. Interestingly the highly variable number of vacant endplates identified in the 4th lumbrical shown in (B), is not reflected in the number of partially occupied endplates (note the different x-axis scale).

(Fig 2.5). The latter was determined by the presence of regular striations (using phase-contrast microscopy) and by no noticeable increase in the levels of autofluorescence that would indicate loss of fibre integrity. No changes to the post-synaptic endplates and muscle fibres are in agreement with previous *in vivo* studies examining the effects of tourniquet induced ischaemia-reperfusion injury (Tombol *et al.* 2002).

2.3.4 Significant loss of α -motor nerve terminal morphology following hypoxia-reperfusion injury.

2H-2R caused $82.5 \pm 4.2\%$ of endplates to appear with no opposing NF/SV₂ immunoreactivity in their motor nerve terminal arborisations and pre-terminal axons (Fig 2.7). The remaining endplates either appeared to show ‘full’ ($16.1 \pm 4.0\%$) or ‘partial’ ($1.4 \pm 0.3\%$) innervation by some nerve terminal boutons. While distal pre-terminal axons were not present, larger branches of the intramuscular nerve could always be identified (Fig 2.4). This dramatic and highly significant loss of nerve terminal morphology was in stark contrast to α -motor nerve terminals from control lumbrical muscles where $99.3 \pm 0.2\%$ of endplates were fully occupied and only a small number of endplates appeared vacant $0.6 \pm 0.2\%$ or partially $0.2 \pm 0.06\%$ occupied.

2.3.5 α -motor nerve terminal undergo rapid disassembly in response to hypoxia-reperfusion injury with characteristics of both withdrawal and Wallerian degeneration –like processes.

Partially innervated endplates were very rarely observed in control preparations but formed a small ($1.4 \pm 0.3\%$) but significant subpopulation in response to 2H-2R (see Fig 2.6-2.7). This offered an ideal opportunity to examine nerve terminals at ‘intermediate’ stages of breakdown and may provide possible insights into the pathways responsible for loss of α -motor nerve terminal morphology. Examination of this subpopulation of neuromuscular junctions revealed an apparently heterogeneous set of morphological responses. Some partially occupied endplates had lost part of their overlying nerve terminal in an asynchronous manner while their remaining nerve terminal and incoming pre-terminal axon remained intact. This is indicative of a withdrawal or retraction-like process of nerve terminal loss (Fig 2.6(B); Gillingwater *et al.* 2002; Gillingwater and Ribchester 2003; Walsh and Lichtman 2003; Parson *et al.* 2004; Bettini *et al.* 2007). Other partially occupied endplates had fragments of remaining nerve terminals that were disconnected from each other and/or their pre-terminal axon (Fig 2.6(A&C)). These fragments were identified by intense NF/SV₂ immunoreactivity and care was taken to ensure that they corresponded with endplate staining to help rule against the possibility of these fragments being non-specific binding artefacts. Fragmentation of axons and nerve terminals is indicative of a classic Wallerian degeneration-like process of nerve terminal loss (Waller

1850; Miledi and Slater 1970; Winlow and Usherwood 1975; Gillingwater and Ribchester 2001; Gillingwater *et al.* 2002; Gillingwater *et al.* 2003). Other α -motor nerve terminals showed aspects of both mechanisms within the same arborisation (Fig2.6C). With evidence of withdrawal and Wallerian degeneration-like process present, it appears unlikely that rapid and significant loss of NF/SV₂ immunoreactivity in α -motor nerve terminals following hypoxia-reperfusion injury occurs by a single mechanism. Furthermore, the overall small population of endplates observed to be partially occupied following 2H-2R indicates that once mechanisms of disassembly are activated, they are rapidly executed.

2.3.6 The 4th deep lumbrical muscle shows a highly variable response to hypoxia-reperfusion injury.

It was noted during data collection that there may be differences in α -motor nerve terminal responses to 2H-2R injury between the four lumbrical muscles (Fig2.7B&C). Data presented above was reanalysed to show the response of α -motor nerve terminals according to the anatomical location/number (Fig 2.1). This revealed that there was a difference, but not statistically significant, in the mean number of vacant endplates in the 4th lumbrical muscle (mean 56.5%) compared to the 1st, 2nd and 3rd lumbricals (collective mean range 89.3% to 91.7%). The 4th lumbrical muscle also showed a very high degree of variance in the number of vacant endplates, ranging from 6.6% to 97% compared to the collective variance of the 1st, 2nd and 3rd lumbrical muscle of 73.6% to 100% (see Fig 2.7B and the appendix of data tables). Further analysis shows that this variance in the number of vacant endplates in the 4th lumbrical was not reflected in the number of partially innervated endplates, which represent ongoing nerve terminal disassembly (Fig 2.7C). This collectively suggests that while the 4th lumbrical has no statistical difference in overall response to hypoxia reperfusion injury, the 4th lumbrical muscle has a greater range of resistance/vulnerability but the cellular processes that govern the loss of α -motor nerve terminal morphology remain unchanged. The reason(s) for this difference remains unclear.

2.3.7 Perturbations to α -motor nerve terminal microtubule network and cytosol following hypoxia-reperfusion injury

The above data clearly shows significant loss of the neurofilament immunoreactivity in α -motor nerve terminals from lumbrical muscles following injury. To investigate further the affect of 2H-2R on the cytoskeletal architecture of α -motor nerve terminals, antibodies directed against the microtubule network, neuron specific β -tubulin III (tubulin), were used following 2H-2R and compared to that of controls. In control preparations tubulin immunoreactivity was intense, without any breaks, in axons and the central regions of the α -motor nerve terminal arborisation, with staining becoming slightly less intense towards the edges of the arborisation ($N=2$, $n=6$: Fig 2.8). Following 2H-2R, tubulin

immunoreactivity became disorganised and obviously fragmented in both axons and α -motor nerve terminals ($N=2$, $n=6$). Unlike NF/SV₂ immunoreactivity where >82% of α -motor nerve terminal appeared vacant, most endplates did appear with some overlying β -tubulin III immunoreactivity but this could sometimes be 'ghostly' in appearance. This would clearly suggest that 2H-2R triggers significant disruption to microtubule network as well as loss of neurofilaments and synaptic vesicles but not complete, gross structural loss of α -motor nerve terminals. This indicates that there are specific spatiotemporal events occurring in response to 2H-2R where neurofilaments and synaptic vesicles are lost prior to microtubules. This is further supported by evidence from α -motor nerve terminals triple-labelled with SV₂, BTX and anti-choline acetyltransferase (Fig2.9). Choline acetyltransferase (CHAT) has a predominantly cytoplasmic pattern of expression (Smith and Carroll 1980; Pahud *et al.* 1998) and its immunoreactivity is routinely used to identify cholinergic neurons in the CNS but has rarely been used to identify α -motor nerve terminals in the periphery. In control conditions CHAT can be seen to intensely stain axons and to co-localise with SV₂ at α -motor nerve terminals and to fully oppose BTX/endplate staining (Fig2.9A: $N=3$, $n=12$). Following 2H-2R (Fig 2.9B: $N=3$, $n=12$), SV₂ staining is lost from most α -motor nerve terminals as above whereas CHAT immunoreactivity largely remains but, on occasion could appear to be fragmented. As CHAT is predominantly cytoplasmic, these findings indicate that while there is loss of neurofilaments, synaptic vesicle proteins and disruption to the microtubule network, the gross structure of α -motor nerve terminals is not lost in response to 2H-2R. In summary, this would suggest that α -motor nerve terminals do not undergo a gross structural loss in response to 2H-2R but are in the process of disassembly with a distinct set of spatiotemporal morphological events.

2.3.8 Selective ultrastructural pathology to nerve terminals in response to hypoxia-reperfusion injury

To help understand some of the morphological events observed above, preliminary ultrastructural analysis was carried out on the 2nd deep lumbrical ($n=1$), as this lumbrical muscle showed the most constant response to 2H-2R (see Fig 2.7B&C). Following 2H-2R (Fig 2.11), muscle fibres appeared healthy with distinctive and regular striations, and the postsynaptic apparatus appeared as in control conditions (Fig2.10). α -motor nerve terminal boutons however were dominated by large vacuoles and disruption of mitochondria. Many axon profiles appeared normal but some also appeared to be undergoing some degenerative events with the cytoplasm becoming very electro-dense. While much further analysis is needed, these preliminary ultrastructural results appear to confirm that α -motor nerve terminals are highly and selective vulnerable to 2H-2R with no observable changes to postsynaptic endplates or muscle fibres. They also support the finding that while α -motor nerve terminals remain grossly intact following 2H-2R, there are distinct pathological events affecting the internal organisation.

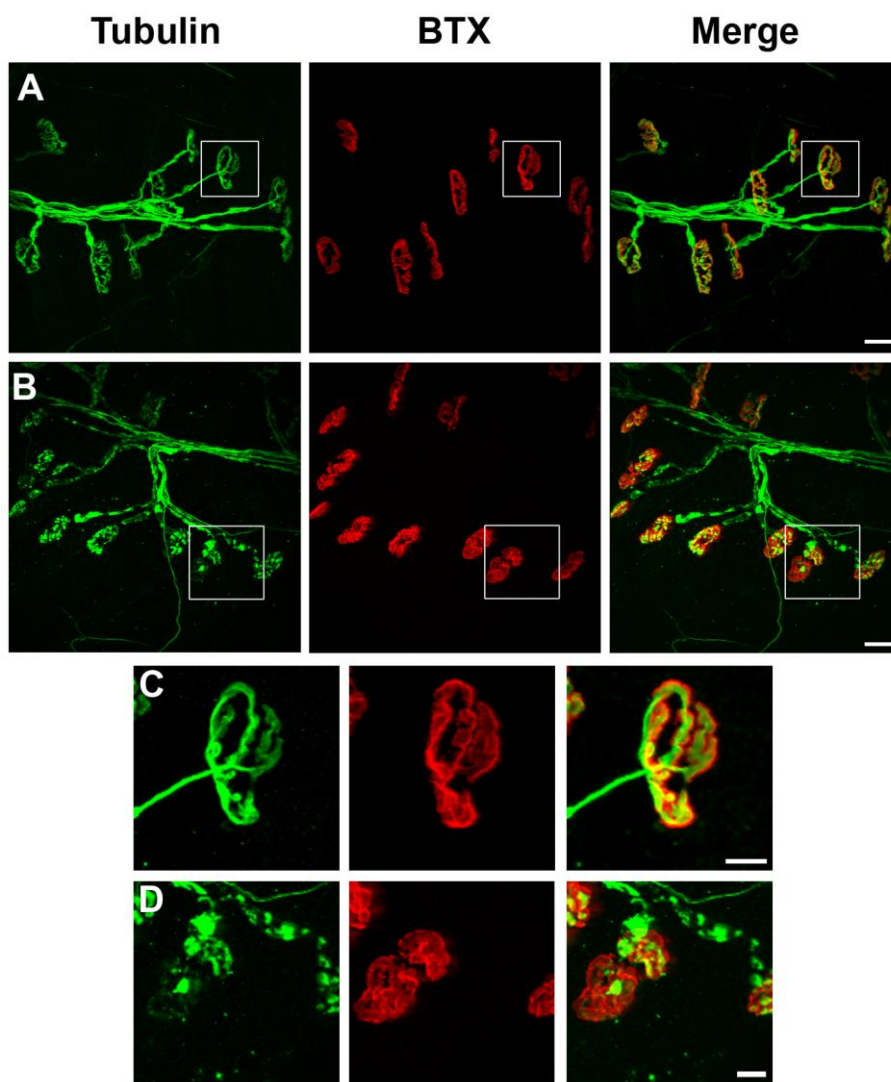


Fig 2.8: Disruption of the microtubule network following hypoxia-reperfusion injury. Confocal projections show neuron specific β -tubulin III visualised with Cy2 secondary antibodies (GREEN) and post-synaptic endplates visualised with TRITC-conjugated BTX (RED). In control condition (**A**), with boxed area magnified in (**C**), strong tubulin immunoreactivity can be seen in both axons and in the central regions of the α -motor nerve terminal arborisation. Slightly less intense tubulin immunoreactivity can be seen to fill the outer regions of the terminal arborisation, but this fully opposes postsynaptic endplates. Following 2H-2R (**B**), with boxed area magnified in (**D**), tubulin immunoreactivity in axons and α -motor nerve terminals can still be identified but is highly fragmented with some regions only just identifiable by low intensity, 'ghostly', immunoreactivity. Scale bars, 20 μ m (A&C), 10 μ m (B&D).

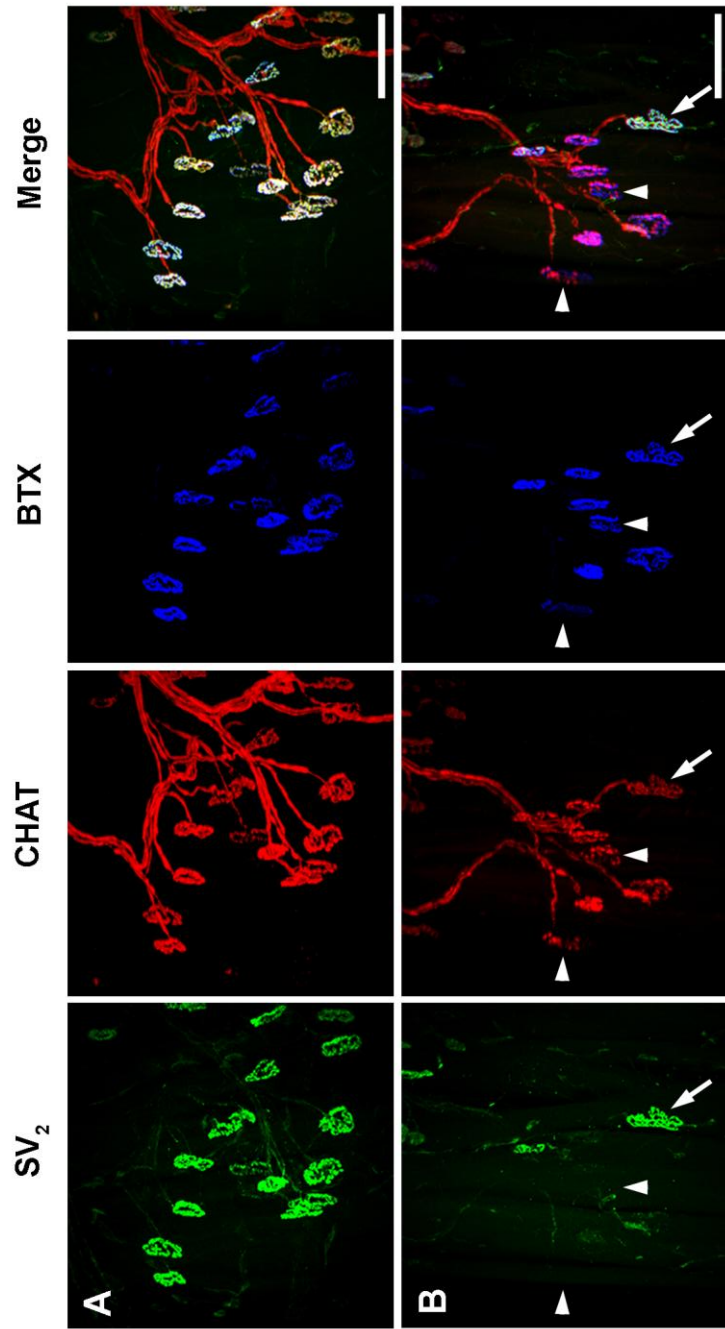


Fig 2.9: Triple-labelled NMJs show loss of synaptic vesicle protein immunoreactivity before changes to gross cytoplasmic structure. Preparations were labelled with antibodies raise against synaptic vesicle protein 2 (SV₂, visualised with anti-mouse Cy-2 secondary antibodies: GREEN), Choline Acetyltransferase (CHAT, visualized with anti-goat Cy-3 secondary antibodies: RED) and with alexa647-conjugated BTX (BLUE) to visualize post-synaptic endplates. Under control conditions (**A**) confocal projections show CHAT immunoreactivity in axons and α -motor nerve terminals to be clear and intense, fully overlying post-synaptic endplates. CHAT also appears to localize well with SV₂ immunoreactivity in α -motor nerve terminals. Faint/diffuse SV₂ staining can also be detected in pre-terminals axons, consistent with vesicles being transported to nerve terminals. Overlaying the images of CHAT, SV₂ and BTX staining causes NMJs to appear white (merge). In response to 2H-2R (**B**), most but not all (**arrow**) α -motor nerve terminals have lost SV₂ immunoreactivity but CHAT immunoreactivity can still be seen to overly all postsynaptic endplates and pre-terminals axons are identifiable. There is some suggestion however, of CHAT staining becoming heterogeneous and possibly fragmented (**arrowheads**) in a nerve terminal that has completely lost its SV₂ immunoreactivity. The loss of SV₂ in most nerve terminals can be clearly seen in the merge image where all but one or two (**arrow**) NMJs appears with a full complement of CHAT, SV₂ and BTX staining and therefore appears white as in controls. The remainder appear purple. Scale bar 50 μ m.

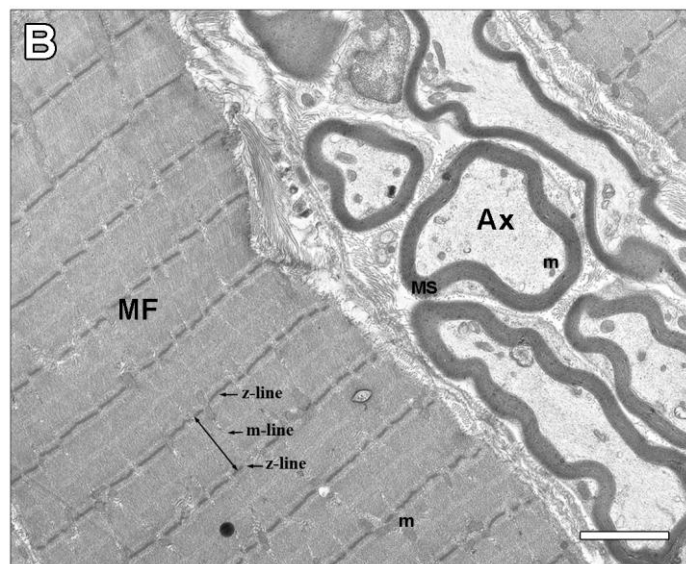
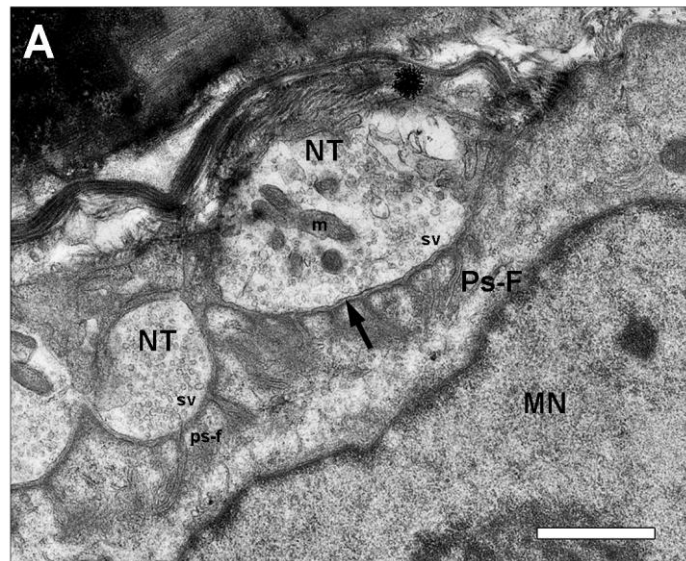
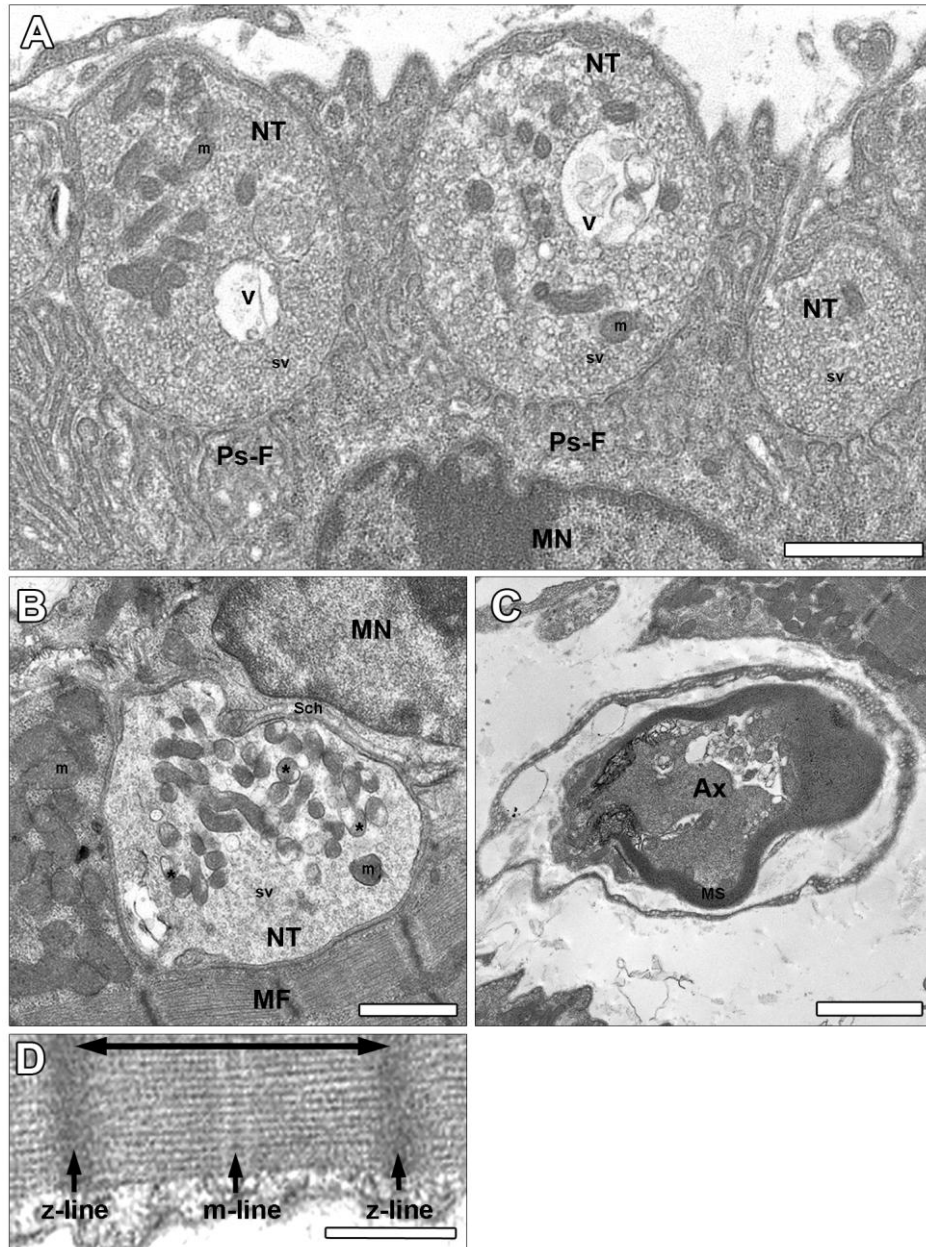


Fig 2.10: Ultrastructure of the 2nd lumbrical muscle kept under control conditions.

In (A), two α -motor nerve terminal boutons (NT) can be observed opposing some postsynaptic folds (Ps-F) separated by the synaptic cleft (arrow). Numerous mitochondria (m) can also be observed with cristae and synaptic vesicles (sv) can be easily identified, ~40nm in size. While the central nerve terminals bouton does appear slightly depleted of vesicles, the nerve terminal bouton in the left of image shows large numbers of vesicles that are clustering towards the membrane. A large muscle fibre nucleus (MN) can also be observed. In (B), a small branch of a small intra-muscular nerve can be identified by numerous axon (Ax) profiles enclosed in a myelin sheath (ms: note how the axo-plasm appears quite electro-lucent). The striations of muscle fibre (MF) are clearly visible with identifiable sarcomeres (arrow), m-lines and z-lines. Numerous mitochondria (m) can be observed to be scattered throughout axon profiles and the muscle fibre. Scale bar 1 μ m (A), 2 μ m (B).



2.11: Selective ultrastructural changes to α -motor nerve terminals and axons from the 2nd lumbrical muscle following hypoxia-reperfusion injury. In (A) several nerve terminal bouton (NT) can be observed with two boutons appearing with large vacuoles (V). In general, boutons appear very electro-dense and while synaptic vesicles (sv) can be identified, they appear irregular and possibly interspersed with small vacuoles especially in the central bouton. The muscle fibre nucleus (MN) can be identified as well as postsynaptic folds (Ps-F) which appear normal and as in controls. Numerous examples of mitochondria (m) can be seen and in (B), many appear disrupted (*) while those in the adjacent muscle fibre (MF) appear normal with clear cristae. A clear Schwann cell process (Sch) can be observed overlying the terminal bouton. In (C), an electro-dense axon profile (Ax) surrounded by the myelin sheath (ms) can be observed while others appeared normal and as in controls. Muscle fibres appeared unaffected by 2H-2R with regular striations (C) and in (D), a sarcomere (arrow) can be seen with regular z- and m-lines. Scale bar 1 μ m (A&B), 2 μ m (C), 0.5 μ m (D).

2.4 Discussion

Here I describe a novel *ex vivo* model of hypoxia-reperfusion injury and show that adult mouse α -motor nerve terminals of the lumbrical muscle are highly and selectively vulnerable to 2hr hypoxia followed by 2hr reperfusion (2H-2R). The model system used to generate hypoxia successfully produced an O₂ concentration below reported values of skeletal muscle normoxia, i.e., <0.5%, and showed rapid and significant loss of morphology in >82% of α -motor nerve terminals. A complex set of spatiotemporal events is indicated by heterogeneous morphological responses and by the range of partially innervated nerve terminals which show features that have previously been identified as being indicative of Wallerian-like degeneration (Waller 1850; Miledi and Slater 1970; Winlow and Usherwood 1975; Gillingwater and Ribchester 2001; Gillingwater *et al.* 2002; Gillingwater *et al.* 2003 and sections 1.5.1) and withdrawal-like processes of nerve terminal loss (Gillingwater *et al.* 2002; Gillingwater and Ribchester 2003; Walsh and Lichtman 2003; Parson *et al.* 2004; Bettini *et al.* 2007: see also below and sections 1.5.2). Ultrastructural analysis of the 2nd deep lumbrical following 2H-2R also revealed pathology indicative of selective α -motor nerve terminal pathology without perturbations to skeletal muscle fibres or postsynaptic apparatus. This demonstrates that despite the belief that peripheral nerves have a greater metabolic safety factor and are better able to supplement their energy requirements via anaerobic pathways than CNS neurons (Stewart *et al.* 1965; Campa and Engel 1971; Low *et al.* 1985; Zollman *et al.* 1991; Mohler *et al.* 1999), hypoxia-reperfusion injury is a major pathological stimulus at α -motor nerve terminals.

2.4.1 hypoxia-reperfusion causes rapid and selective structural pathology to α -motor nerve terminals

Nerve terminals are known to be highly vulnerable to a range of pathological stimuli (e.g., Raff *et al.* 2002; Wishart *et al.* 2006; Conforti *et al.* 2007a) but there has yet to be a conclusive study that demonstrates the structural vulnerability of α -motor nerve terminals to hypoxia/ischaemia-reperfusion injuries. Previous studies have identified that α -motor nerve terminals may be vulnerable following *in vivo* ischaemia-reperfusion injuries but have failed to clearly distinguish the cause of this from tourniquet induced ischaemia-reperfusion injury, tourniquet induced mechanical trauma or that of muscle damage (Makitie and Teravainen 1977a; Diwan and Milburn 1986; Tombol *et al.* 2002; Eastlack *et al.* 2004; David *et al.* 2007). Other studies have shown that *ex vivo* α -motor nerve terminals are functionally vulnerable to hypoxia-reperfusion injury, but the observed changes were rapidly reversible with no reports of structural pathology (Krnjevic and Miledi 1959; Hubbard and Loyning 1966; Anwyl and Ling 1983; Nishimura 1986; Bazzi 1994; Zhu *et al.* 2006). In the present study, I have shown distinct and selective structural pathology at *ex vivo* α -motor nerve terminals following 2H-2R. It has previously been shown that muscle fibre trauma (Rich and Lichtman 1989;

Bernstein and Lichtman 1999) and disruption to postsynaptic endplates (Balice-Gordon and Lichtman 1994; Rich *et al.* 1994; McCann *et al.* 2007) can all cause loss of α -motor nerve terminals. In all these studies however, α -motor nerve terminal pathology develops over a number of days even *in vivo*. In the present study, α -motor nerve terminals developed significant pathology within 4hr with no evidence of structural damage to postsynaptic endplate or muscle fibres. This is in direct contrast to several studies examining the effects of ischaemia/ischaemia-reperfusion injuries induced by tourniquet or arterial occlusion that have shown α -motor nerve terminal pathology alongside muscle fibre (Makitie and Teravainen 1977a; Diwan and Milburn 1986) and endplate (Hatzipantelis *et al.* 2001) pathology. These results are however in line with those found by Tombol *et al.* (2002) that have shown selective ultrastructural changes, including vacuolisation, synaptic vesicle depletion and loss of α -motor nerve terminal profiles in response 2hr tourniquet followed by ≥ 2 hr-reperfusion injury. What is not clear from the results presented here is whether distal axon pathology is a result of a retrograde spread of α -motor nerve terminal pathology or if axonal pathology is induced by hypoxia-reperfusion injury independently of events at terminals. Axons have been shown to be vulnerable in *in vivo* models of ischaemia-reperfusion injuries (Nukada *et al.* 1996; Iida *et al.* 2003; Kawamura *et al.* 2006) but functional analysis by others has collectively shown that axonal conduction (Fish *et al.* 1989; Zollman *et al.* 1991; Pedowitz *et al.* 1992; Ohara *et al.* 1996; Mohler *et al.* 1999) and skeletal muscle (Krnjevic and Miledi 1959; Hubbard and Loyning 1966; Chervu *et al.* 1989; Bazzzy 1994; Hatzipantelis *et al.* 2001; Zhu *et al.* 2006) recover, which indicates the primary site of injury to be at the NMJ. The preservation of NF/SV₂ immunoreactivity in the large intramuscular nerve branches (Fig 2.4) but loss of α -motor nerve terminal NF/SV₂ immunoreactivity in response to 2H-2R presented here would certainly indicate a distal to proximal progression. This collectively suggests that pathology begins in α -motor nerve terminals but further studies are needed to confirm this. Additional experiments that include functional analysis may be beneficial in resolving this issue and could be used to determine if 2H-2R also causes functional deficits in the muscle fibres and endplates. While the results obtained here indicate that muscle fibres and postsynaptic endplate were not structurally vulnerable to 2H-2R, functional changes to either cannot be ruled out, as this was not studied.

2.4.2 Pathology is primarily caused by changes in O₂ levels

I have demonstrated that structural pathology occurs as the result of O₂ level being less than 0.25%, which replicates the O₂ levels that have previously been reported to occur during *in vivo* ischaemia (Gustafsson *et al.* 1999; Matsumoto *et al.* 2005). This is in contrast to previous *ex vivo* studies of hypoxia-reperfusion injury where O₂ levels at the muscle surface or just below have been recorded as being between 6% and 8% (Bukharaeva *et al.* 2005; Zhu *et al.* 2006) while others have recorded intramuscular values of 0% to 2.6% (Bazzzy 1994). As the normoxic working range of skeletal muscle, and probably α -motor nerve terminals, has been estimated to be between 0.5% and 4% (Gorczyński

and Duling 1978; Klitzman *et al.* 1983; Honig and Gayeski 1993; Shibata *et al.* 2001; Eu *et al.* 2003; Matsumoto *et al.* 2005), some of these studies may not have been modelling an hypoxic environment. While it can be expected that muscle fibres and their nerve terminals located in the central, deeper regions are subject to much lower levels of O₂ due to the reliance on diffusion (Eu *et al.* 2003; Zhu *et al.* 2006), the result presented here show that even those muscle fibres and α -motor nerve terminals located on the surface of the muscle are subject to hypoxic conditions. In contrast to previous *in vivo* studies that cannot distinguish between the range of potentially pathological co-stimuli caused by ischaemia-reperfusion injuries, I show that the α -motor nerve terminal pathology was almost solely attributable to changes in O₂. Acidosis, known to occur in O₂ limiting conditions (Cerretelli and Samaja 2003) contributes significantly to CNS hypoxia/ischaemia-reperfusion injuries (Kass and Lipton 1982; Fujiwara *et al.* 1992; Nagao *et al.* 1996) and in the periphery (Silver 1977; Kam *et al.* 2001) was prevented in the current study. Significant changes in CO₂ levels, also known to occur during ischaemic injuries (Rorabeck 1980; Kam *et al.* 2001; Kam 2007) can also contribute to acidification are also unlikely in the model system presented here as levels were maintained at 5%. A gross systemic inflammatory response occurs in CNS (Nogawa *et al.* 1997; Samdani *et al.* 1997; Fukuyama *et al.* 1998; Lipton 1999; Costa *et al.* 2006; Doyle *et al.* 2008; Froyland *et al.* 2008) and in peripheral ischaemia-reperfusion injuries (Carden and Granger 2000; Kam 2007; Bagdatoglu *et al.* 2008) is also unlikely to have significantly contributed to the pathology described here due to the reduced nature of neuromuscular preparations and the rapidity of the response. The relatively large volume of perfusate (250ml) containing glucose should have also prevented hypoglycaemia as well reducing the build up of potentially toxic extracellular metabolites and extracellular ionic changes that have all been identified to occur during *in vivo* ischaemia-reperfusion injuries (e.g., Rorabeck 1980; Idstrom *et al.* 1990; Pedowitz *et al.* 1992; Nagamatsu *et al.* 1996; Kam *et al.* 2001). I also demonstrate that α -motor nerve terminal pathology induced by 2H-2R was probably independent of axotomy/lesion induced Wallerian degeneration. α -motor nerve terminals were protected from Wallerian degeneration by long distal nerve stumps (>2.5cm) that provide 1-2hr protection per cm (Miledi and Slater 1970; Ribchester *et al.* 1995) and by low temperatures (22-24.5°C) which delay Wallerian degeneration by up to 4 times at 25°C (Tsao *et al.* 1999). Furthermore, total experimental time did not exceed 5.5hr (~1hr to complete dissection + 4hr hypoxia-reperfusion injury + time to complete BTX staining and fixation), and the first signs of Wallerian degeneration *in vivo* begin at around 12hr (Miledi and Slater 1968; 1970; Winlow and Usherwood 1976; Tsao *et al.* 1999; Gillingwater and Ribchester 2001). While some possible contribution of Wallerian degeneration resulting from axotomy cannot be entirely ruled out, others have used similar neuromuscular preparations over >4-6hr and shown axotomy independent changes at α -motor nerve terminals (Parson *et al.* 2004; Bettini *et al.* 2007). This contrasts with many *in vivo* studies of ischaemia reperfusion injury that have utilised tourniquets (Fowler *et al.* 1972; Makitie and Teravainen 1977b; Nukada and McMorran 1994; Eastlack *et al.* 2004) and include using elastic rubber bands wound round the hind limbs (Tombol *et al.* 2002; David *et al.* 2007) that have significant risk of causing

lesions to underlying nerves. There is some difficulty distinguishing the cause of α -motor nerve terminal pathology identified in some of these *in vivo* studies from ischaemia-reperfusion injury and axotomy/lesion induced Wallerian degeneration especially when experimental procedure was in excess of 12hr. This study therefore demonstrates that hypoxia-reperfusion injury is a significant pathological stimulus at α -motor nerve terminals and can induce rapid structural pathology.

2.4.3 Which aspects of changing O₂ levels are causing α -motor nerve terminal pathology?

What is unclear from the model presented here is what aspects of the hypoxic or the reperfusion stages are contributing to α -motor nerve terminal pathology. Hypoxia inhibits oxidative phosphorylation causing ATP depletion that leads to a failure to meet the bioenergetic demands required to maintain cellular homeostasis, morphology, and to carry out generic as well as cell-specific functions. ATP depletion/bioenergetic rundown have been shown to occur as a result of hypoxic/ischaemic injury in peripheral nerve bundles (Stewart *et al.* 1965; Low *et al.* 1984; Zollman *et al.* 1991) and are well characterised in CNS injuries (Dewar *et al.* 1999; Lipton 1999; Ames 2000; White *et al.* 2000; Bickler and Donohoe 2002). As nerve terminals are known to have high energetic demands (Wong-Riley 1989; Miller and Sheetz 2004; Hollenbeck and Saxton 2005) but the mitochondria contained within them appear to be more susceptible to inhibition and rupture (John 1996; Davey *et al.* 1998; Brown *et al.* 2006; Naga *et al.* 2007), overall it seems likely that ATP depletion as a result of hypoxia is the major pathological stimulus in the results presented here. This is further supported by observations of disruption of mitochondrial ultrastructure in response to 2H-2R (Fig2.11B), but further studies are needed to show if this results in ATP depletion. What the consequences of this ATP depletion are and how that leads to the observed pathological response in α -motor nerve terminals is unknown and requires further study. For example, one of the major stimuli caused by ATP depletion is breakdown of Na⁺, K⁺, Ca²⁺, Fe²⁺ and H⁺ ionic gradients which are critical in mediating CNS ischaemic injuries (e.g., depolarisation and excitotoxicity: e.g., Lipton 1999). While extracellular ionic changes were probably prevented by the large volume of perfusate in this study, intracellular changes are still likely to have occurred, including increases in Ca²⁺ (possibly, but not only from disrupted mitochondria), Na⁺ and acidosis. Furthermore, extracellular changes cannot be ruled out in poorly diffused microdomains, such as synaptic cleft. Carrying out experiments in the presence of glucose may also have contributed to acidification, as any continuing respiration via anaerobic glycolysis would be associated with lactate production and increasing acidification (Ridge 1972; Lipton 1999; Cerretelli and Samaja 2003; Malthankar-Phatak *et al.* 2008). Reoxygenation/reperfusion injury is also known to contribute significantly to pathology in both the CNS and peripheral nerves. Reperfusion allows for minor cellular insults caused during the hypoxic/ischaemic phase to develop and manifest into late-stage pathology, and in both the CNS (Nakano *et al.* 1990; Lipton 1999; Kovalenko *et al.* 2006) and peripheral nerve bundles (Nukada *et al.* 1996; Iida *et al.* 2003; Kawamura *et al.* 2006) the full extent of pathology is not seen until days or even weeks after the initial ischaemic insult. On the other hand,

reperfusion is also associated with its own pathological stimuli. Once blood flow or O₂ levels are fully restored, the return of O₂ is known to trigger significant formation of ROS due to clearance of toxic metabolites (e.g., adenine nucleotide breakdown products via xanthine oxidase: Nagamatsu *et al.* 1996; He *et al.* 1999; Mitsui *et al.* 1999c), activation of inflammatory mediators (Mitsui *et al.* 1999a; Qi *et al.* 2001; Kawamura *et al.* 2006), but also damage to or disequilibrium of the mitochondrial respiratory chain (Piantadosi and Zhang 1996; Nicholls and Budd 2000). Although not directly shown but discussed above, extracellular ionic changes, build-up of potential toxic metabolite and systemic inflammation have been largely (but not entirely) ruled out in this model system. Re-oxygenation was however carried out at ~50% O₂ (presumably reflecting the O₂ saturation point for Krebs' solution sparged with 95% O₂ gas), well above *in vivo* level of normoxia, possibly increasing the risk of ROS formation. This would overall indicate that the reperfusion stage of the model presented here is a weak paradigm of pathological stimuli associated with *in vivo* reperfusion injuries. There is however, the general difficulty in all studies examining ischaemia/hypoxia-reperfusion injuries: how do you distinguish between pathology that was caused by the hypoxic/ischaemic injury but has taken time to develop/become identifiable, from the pathology caused by the reperfusion injury? Nevertheless, using the model system developed here, others have recently provided preliminary evidence to show that a significant amount of pathology can be caused by hypoxia alone, with 23% and 44% of postsynaptic endplates from lumbrical muscles appearing vacant following 1.5hr and 2hr hypoxia respectively (preliminary observations made during undergraduate BSc research project/dissertation undertaken by Miss Marie Parsons (2008) at the University of Edinburgh under the supervision Simon Parson). Overall, this study does demonstrate that changes in O₂ tension during hypoxia-reperfusion injury is a major pathological stimulus at α -motor nerve terminals however, more studies are needed to understand further the cellular and biochemical events that mediate this injury. These studies should include assessment of the biogenetic changes following hypoxia-reperfusion injury, as well as intra and extra-cellular ionic changes. The robust nature of the model system described here will be of benefit in such studies as many external variables can be controlled and it will also allow for systematic pharmacological investigations that would help in our understanding the underlying mechanisms of hypoxia-reperfusion injury.

2.4.4 α -motor nerve terminal pathology is indicative of on-going disassembly

Synaptic vesicles, neurofilaments and microtubules are vitally important features of α -motor nerve terminals and the loss or disruption of immunoreactivity in response to 2H-2R indicates significant impairment to the mechanical stability, transport and function of α -motor nerve terminals. The degradation and loss of neurofilaments, microtubules and synaptic vesicles in α -motor nerve terminals and axons are known to occur in response to other neurodegenerative processes including axotomy-induced Wallerian degeneration and in peripheral neuropathies (e.g., Miledi and Slater 1970; Miura *et al.* 1993; Gillingwater *et al.* 2002; Ferri *et al.* 2003; Gillingwater and Ribchester 2003; Zhai *et al.*

2003; Parson *et al.* 2004; Pun *et al.* 2006; Wishart *et al.* 2006; Bettini *et al.* 2007; Murray *et al.* 2008a). These studies have shown that progressive loss of neurofilament and/or synaptic vesicle protein immunoreactivity is not only a good correlate of nerve terminals loss but also a good indicator of the mechanism of loss, either Wallerian degeneration or withdrawal (e.g., Gillingwater *et al.* 2002; Gillingwater and Ribchester 2003; Parson *et al.* 2004; Bettini *et al.* 2007). But, the results presented here indicate that the gross structure of α -motor nerve terminals is not lost following 2H-2R as α -motor nerve terminal profiles could be still identified with antibodies raised against tubulin, cytoplasmic CHAT and observed at the ultrastructural level, although some pathological changes were noted. Loss of neurofilaments but not microtubules has however been shown to occur during ongoing Wallerian degeneration in primary cultures of rat sympathetic superior ganglia (Zhai *et al.* 2003). These authors have shown that 4-8hr following axotomy, microtubules become depolymerised/disorganised (LoPachin *et al.* 2002; LoPachin *et al.* 2003) and this is followed by degradation of neurofilaments but, microtubule degradation and loss of immunoreactivity did not occur until >16hr post-axotomy. It is possible that a similar set of spatiotemporal events are also occurring in response to 2H-2R, and that while numerous authors have shown α -motor nerve terminal loss with antibodies raised against NF/SV₂ or similar (e.g. Trachtenberg and Thompson 1997; Dixon and Harris 1999; van der Putten *et al.* 2000; Gillingwater *et al.* 2002; Parson *et al.* 2004; Prasarnpun *et al.* 2004; Yin *et al.* 2004; Bettini *et al.* 2007; Murray *et al.* 2008a) this may not always completely coincide with complete gross structural loss. The results presented here most likely reflect a process of α -motor nerve terminal and distal axon disassembly that may eventually lead to gross structural loss where it could be expected to see loss of tubulin and cytoplasmic CHAT immunoreactivity as disassembly progresses to loss. In future studies, it would be particularly important to determine when α -motor nerve terminals pathology first develops and if, and when, pathology advances into gross structural loss. In addition, combinations of a broader spectrum of immunohistochemical markers labelling a range of different membranous, cytoskeletal and vesicular proteins, alongside a complete ultrastructural analysis as well as the functional analysis already suggested would be beneficial in developing our understanding of the pathological events of hypoxia-reperfusion injury.

2.4.5 Does hypoxia-reperfusion cause multiple mechanisms of α -motor nerve terminal disassembly?

A review of the literature clearly provides evidence of at least two structurally and mechanistically distinct processes of nerve terminal loss/disassembly: the synchronous fragmentation of Wallerian degeneration and asynchronous retraction of withdrawal (Bernstein and Lichtman 1999; Gillingwater and Ribchester 2001; Raff *et al.* 2002; Gillingwater and Ribchester 2003; Low and Cheng 2005; Luo and O'Leary 2005; Saxena and Caroni 2007; Murray *et al.* 2008b). In the results presented here there was a small (1.4%) but significant population of α -motor nerve terminals with NF/SV₂ staining

characteristics of both Wallerian degeneration and withdrawal to suggest multiple mechanisms of nerve terminal loss are triggered in response to 2H-2R.

Is there evidence to suggest that Wallerian degeneration and/or withdrawal could be triggered in response to hypoxia/ischaemia-reperfusion injuries?

Wallerian degeneration appears to be a global means of axonal and nerve terminal loss, triggered by a diverse range of stimuli (Raff *et al.* 2002; Ferri *et al.* 2003; Coleman 2005; Luo and O'Leary 2005; Saxena and Caroni 2007) such as; disruption of microtubules (Luduena *et al.* 1986; Wang *et al.* 2000; Wang *et al.* 2001), progressive motor neuropathy (Ferri *et al.* 2003; Simonin *et al.* 2007), genetic dysmyelination disorder (Samsam *et al.* 2003), axonal dystrophy (Mi *et al.* 2005), autoimmune encephalomyelitis (Kaneko *et al.* 2006), traumatic brain injury (Gillingwater *et al.* 2006a), glaucoma (Howell *et al.* 2007; Beirowski *et al.* 2008), Parkinson's diseases (Sajadi *et al.* 2004; Hasbani and O'Malley 2006), removal of NGF (Deckwerth and Johnson 1994). The *Wld^s* mutation has also been shown to convey some neuroprotection in CNS ischaemia-reperfusion injuries, demonstrating that Wallerian degeneration plays an important role in these types of injuries (Gillingwater *et al.* 2004) and therefore may be triggered in α -motor nerve terminals in response to 2H-2R. Degeneration of α -motor nerve terminals following *in vivo* ischaemia via Wallerian-like pathways has also been previously suggested (Diwan and Milburn 1986). Further support for activation of Wallerian degeneration pathways is provided by studies that show the decreasing levels of bioenergetic molecules, including ATP, appear critical in mediating this mechanism (Coleman 2005). Following injury and during the lag phase prior to the onset of Wallerian degeneration, there is a progressive fall in levels of ATP and NAD⁺ (Stewart *et al.* 1965; Ikegami and Koike 2003; Wang *et al.* 2005). If the levels of bioenergetic molecules, including ATP, are maintained by application of NAD⁺, in neurons over expressing Nmant1 (a NAD⁺ biosynthetic enzyme) or the *Wld^s* mutation that has Nmant1 as part of its protein product, Wallerian degeneration is inhibited (Mack *et al.* 2001; Ikegami and Koike 2003; Araki *et al.* 2004; Wang *et al.* 2005; Kaneko *et al.* 2006; Sasaki *et al.* 2006; Sasaki *et al.* 2009b; Yahata *et al.* 2009). This indicates that a fall in ATP is vital for initiating Wallerian degeneration and therefore, decreasing ATP levels as a consequence of hypoxia-reperfusion would be consistent with activation of Wallerian degeneration.

Withdrawal appears to be triggered by local stimuli and in particular, by loss of/changes in trophic support (e.g., Balice-Gordon and Lichtman 1994; Trachtenberg and Thompson 1997; Bernstein and Lichtman 1999) and if it is under the active control of actin cytoskeleton as has been suggested, may be a highly dynamic and reversible process (Billuart *et al.* 2001; Luo and O'Leary 2005; Vega-Riveroll *et al.* 2005; Saxena and Caroni 2007). In line with the view that withdrawal or "atrophy" of nerve terminals is triggered by loss of/changes in trophic support (Bernstein and Lichtman 1999), depletion of ATP (that is vital for all cell processes) could conceivably cause atrophy/withdrawal of α -motor nerve terminals in response to 2H-2R. There is a possibility that ATP depletion causes a loss of other trophic support/signalling processes provided by the motor neuron itself (i.e., cell body),

muscle fibres or Schwann cells due to inhibition of axonal transport and protein synthesis. Considering the rapid disassembly of α -motor nerve terminals observed here, it seems unlikely that there was sufficient time for trophic support/signals to have become depleted and to then trigger α -motor nerve terminal disassembly, as all previous studies demonstrate this type of trophic deprivation takes a number of days (Rich and Lichtman 1989; Balice-Gordon and Lichtman 1994; Trachtenberg and Thompson 1997; Bernstein and Lichtman 1999; McCann *et al.* 2007; Murray *et al.* 2008b). Alternatively, if withdrawal is an active withdrawal mechanism mediated by the actin cytoskeleton (Billuart *et al.* 2001; Luo and O'Leary 2005; Vega-Riveroll *et al.* 2005; Saxena and Caroni 2007) that is repressed under normal physiological conditions (Billuart *et al.* 2001; Lamprecht *et al.* 2002), ATP depletion may cause a failure in repression of the retraction mechanism. In a recent study that demonstrated rapid (>4hr) withdrawal of adult mouse α -motor nerve in response P2X₇ –receptor activation, the authors also proposed a mechanism that may be important in mediating ischaemic injuries (Bettini *et al.* 2007). P2X₇ –receptors are ligand-gated non-selective cation channels (Surprenant *et al.* 1996; Chessell *et al.* 1998) that are known to be expressed at nerve terminals (Deuchars *et al.* 2001; Moores *et al.* 2005; Anderson and Nedergaard 2006), and have been linked with downstream modifications to the actin cytoskeleton (Kim *et al.* 2001; Pubill *et al.* 2001; Omatsu-Kanbe *et al.* 2004; Pfeiffer *et al.* 2004). These receptors are thought to primarily have a patho-physiological role as they are only opened by high concentrations of their endogenous ligand, ATP (North and Surprenant 2000; North 2002; Burnstock 2006). At nerve terminals, extracellular accumulation of ATP can occur in response to vesicle release (Silinsky 1975) as, independent of its bioenergetic role, ATP is a co-transmitter (Abbracchio *et al.* 2008) and in α -motor nerve terminals it is stored alongside ACh in an estimated ATP:ACh ratio of between 1:1 to 1:10 (Volkhardt and Zimmermann 1986; Smith 1991). It is known that excitotoxicity is a major pathological stimulus in CNS hypoxia/ischaemia-reperfusion injuries (Rothman 1983; Rothman 1984; Lipton 1999; White *et al.* 2000) and significant increases in vesicle release have previously been shown at α -motor nerve terminals in response to hypoxia (Krnjevic and Miledi 1959; Hubbard and Loyning 1966; Anwyl and Ling 1983; Nishimura 1986; Bazzay 1994; Zhu *et al.* 2006). It is possible that during hypoxia/ischaemia-reperfusion injuries such as those observed here, accumulation of ATP at the synaptic cleft because of rapid vesicle release may cause P2X₇-receptor activation leading to rapid withdrawal of α -motor nerve terminals, possibly mediated by the actin cytoskeleton, like that described by Bettini *et al.* (2007). In support of this, the ionic gradient breakdown seen during hypoxic/ischaemic insults would facilitate activation of P2X₇-receptor opening (Colomar and Amedee 2001; North 2002; Moores *et al.* 2005) and P2X₇-receptors have previously been identified to be involved in mediating cell death in neurodegenerative/pathological conditions (Le Feuvre *et al.* 2002; Yiangou *et al.* 2006; Matute *et al.* 2007) and in CNS hypoxia/ischaemia-reperfusion injuries (Le Feuvre *et al.* 2003; Cavaliere *et al.* 2004).

Could necrotic mechanisms play a role in observed responses of α -motor nerve terminals?

There is however, the possibility that despite the morphological appearances, neither Wallerian degeneration nor withdrawal processes are responsible for α -motor nerve terminal disassembly in response to hypoxia-reperfusion injury. The structural events observed in response to LTX and auto-

ganglioside/complement attack causes very rapid (<3hr) necrotic pathology to α -motor nerve terminals that includes breakdown of the cytoskeleton, loss of synaptic vesicles, and, swelling and lysis of nerve terminal boutons (Clark *et al.* 1970; Okamoto *et al.* 1971; O'Hanlon *et al.* 2001; O'Hanlon *et al.* 2003). While the pathology observed in α -motor nerve terminals in response to 2H-2R does not appear to be as extensive as those described in response to these specific necrotic stimuli, the rapid changes to synaptic vesicles and the cytoskeleton is perhaps consistent with some of these necrotic features. Furthermore, necrotic mechanisms of cell death are known to occur in acute hypoxia/ ischaemia reperfusion injuries (Lipton 1999; White *et al.* 2000). It is possible, that due to the relatively large size of α -motor terminals, spatiotemporal activation of singular mechanism, possibly necrotic, gave the morphological appearance of both Wallerian degeneration and withdrawal. This is perhaps supported by a recent structural analysis of the response of α -motor nerve terminals to β -bungarotoxin (Prasarnpun *et al.* 2005). β -bungarotoxin is a necrotic stimulus (Dixon and Harris 1999; Prasarnpun *et al.* 2004; Prasarnpun *et al.* 2005) and shares many similarities with LTX auto-ganglioside/complement attack in that it is a very specific, rapid insult that has Ca^{2+} mediated component and causes lysis of presynaptic membranes (Abe *et al.* 1976; Dixon and Harris 1999; Prasarnpun *et al.* 2004; Prasarnpun *et al.* 2005; Rigoni *et al.* 2008). Prior to this however there is loss of synaptic vesicles and their release machinery (synaptophysin, SNAP and syntaxin), swelling and lysis of mitochondria, and loss of neurofilaments over 3-12hr *in vivo* (Abe *et al.* 1976; Dixon and Harris 1999; Prasarnpun *et al.* 2004; Prasarnpun *et al.* 2005). Prasarnpun *et al.* (2005) noted that some α -motor nerve terminals showed an overall gross morphology that was necrotic, others with obvious signs of fragmentation (Wallerian-like), while others appeared to be withdrawing, very much like the results presented here. This does suggest that perhaps necrosis may also have a role to play in the observed response of α -motor nerve terminals described here and that necrotic stimuli can give the impression of multiple mechanisms of α -motor nerve terminal disassembly. Prasarnpun *et al.* (2005) however believe their results reflect activation of multiple mechanisms of nerve terminal disassembly which is probably due to the multiple molecular mechanisms of β -bungarotoxin toxicity which appears to involve phospholipid hydrolysis, massive Ca^{2+} entry and, a direct action on mitochondria and opening of their pores (Prasarnpun *et al.* 2004; Rigoni *et al.* 2008).

Are transitions between different mechanisms of nerve terminal disassembly responsible for the range of morphologies observed?

There is increasing awareness that the mode of cell death and therefore, perhaps the mode of nerve terminal disassembly is heavily dependent on the availability of ATP (e.g., Ankarcrona *et al.* 1995; Eguchi *et al.* 1997; Leist *et al.* 1997; Fujikawa 2000; Nicotera *et al.* 2000; Northington *et al.* 2007). During and following hypoxic/ischaemic insults ATP levels become increasingly depleted due to increasing levels of cellular damage. Once cellular damage has reached levels sufficient to activate a cell death program, continuing ATP depletion may prevent a cell from completing an active ATP dependent program of cell death and force a more passive form of cell death, resulting in different

morphological phenotypes of cell death with intermediate forms (Fujikawa 2000). There is some debate as to whether this change reflects a switch between multiple independent pathways of cell death (Portera-Cailliau *et al.* 1997; Northington *et al.* 2007) or rather a morphological and mechanistic continuum between necrotic and apoptotic extremes (Yakovlev and Faden 2004). This possibility of changing mechanisms may help to explain the appearance of multiple morphological phenotypes of α -motor nerve terminal loss following hypoxia-reperfusion injury. α -motor nerve terminals may be progressing through withdrawal, Wallerian and possibly necrotic mechanisms of disassembly caused by subtle differences in pathological stimuli and/or changing levels of ATP. This possibility of mechanistic transitions may also be supported by the observations of single α -motor nerve terminals with characteristics of both withdrawal and Wallerian degeneration (Fig 2.6(C)). Other studies examining α -motor nerve terminals during developmental synapse elimination and in response to pathological insults in adults have also noted similar results, with nerve terminals undergoing a process of withdrawal but with some fragmentation and isolation of boutons, and in some cases observations of degenerative ultrastructure (Rich and Lichtman 1989; Trachtenberg and Thompson 1997; van der Putten *et al.* 2000; Gillingwater *et al.* 2003; Bishop *et al.* 2004; Yin *et al.* 2004; Newbery *et al.* 2005; Bettini *et al.* 2007; Ey *et al.* 2007). Some have suggested this duplicity reflects incomplete execution of the withdrawal mechanism with fragments of nerve terminals/axons getting 'left behind' during retraction (Gillingwater and Ribchester 2003) but others have suggested that it may be that shedding of fragments represents a separate mechanism of removing excess neuronal debris (Bishop *et al.* 2004).

Section Summary

From the morphological result presented here, there was evidence to suggest that the two most highly characteristic mechanisms of nerve terminal disassembly, Wallerian degeneration and withdrawal, were triggered in response to hypoxia-reperfusion injury. It must be noted however, the *preliminary* results from the ultrastructural analysis showed domination of α -motor nerve terminal profiles that had significant perturbations to the internal organisation (i.e. vacuoles and mitochondria) whereas withdrawal, at the ultrastructural level, is characterised by minimal internal reorganisation (Gillingwater and Ribchester 2001; O'Hanlon *et al.* 2001; Gillingwater *et al.* 2002; Parson *et al.* 2004; Ushkaryov *et al.* 2004; Bettini *et al.* 2007). From the discussion above however, this preliminary evidence of degenerative ultrastructure may not negate the possibility of the activation of a withdrawal mechanism. A full, and perhaps quantitative, ultrastructural analysis of the response of α -motor nerve terminals would be beneficial in helping to understand the pathological processes of hypoxia-reperfusion injury and may give further insight as to the mechanisms that govern nerve terminal disassembly. Collectively, the evidence supports the possibility that multiple mechanisms of α -motor nerve terminal disassembly could be activated in hypoxia-reperfusion injuries. Multiple mechanisms of cell death characterise CNS ischaemia-reperfusion injuries and neurodegeneration caused by glutamate excitotoxicity where different mechanisms of cell death can be observed between, and

within the same population of neurons (Ankarcrona *et al.* 1995; Eguchi *et al.* 1997; Leist *et al.* 1997; Northington *et al.* 2007; Hand and Menze 2008). The reasons underlying the activation of multiple mechanisms is still not understood but as hypoxia/ischaemia-reperfusion injuries involve so many complex co-stimuli, activation of multiple cellular pathways leading to cell death is perhaps not surprising. The range of possible pathological stimuli following hypoxia-reperfusion injury at α -motor nerve terminals in the present study has purposely been reduced but will still most likely include ATP depletion, dissipation of ionic gradients including Ca^{2+} , direct mitochondrial damage and ROS formation. These stimuli acting independently and/or synergistically may affect each α -motor terminal differently to cause activation of different mechanisms of nerve terminal disassembly. Further work on the molecular mechanisms of α -motor nerve terminal disassembly is required as our understanding of the cellular processes that govern them are still poor. Given the range of morphologies observed in response to hypoxia-reperfusion injury described here, this model system may provide an opportunity to investigate some of these mechanisms. The rapid response, control of pathological stimuli and the ability to apply a range of pharmacological agents as well as to use genetically altered tissues provides the opportunity to study systematically the mechanism(s) of hypoxia-reperfusion injury. This may in turn provide further insights into α -motor disassembly in response to both hypoxia-reperfusion injury but also other pathological stimuli. Of particular interest would be an investigation into the ability of the *Wld^s* mutation to convey neuroprotection to α -motor nerve terminals following hypoxia-reperfusion injury.

2.4.6 Summary

I have demonstrated for the first time that α -motor nerve terminals of the adult mouse are structurally vulnerable to hypoxia-reperfusion injury. With a novel experimental model system, I have shown that the rapid and selective disassembly of α -motor nerve terminals occurs in response to O_2 levels similar to those occurring during *in vivo* ischaemia. I highlight that further studies are needed to fully characterize this model system and to understand the cellular/molecular consequences of hypoxia-reperfusion, which is more than likely a consequence of ATP depletion. While the experimental model system developed here is not without its caveats, it may provide a valuable tool in further studies of α -motor terminal pathology in response to hypoxia/ischaemia-reperfusion injuries but may also provide insights into pathological consequences caused by other stimuli.

Chapter 3

α -motor nerve terminal pathology following hypoxia-reperfusion injury is age and fibre type-dependent but is not mitigated by the neuroprotective *Wld^s* mutation.

3.1 Introduction

In Chapter 2, I showed that α -motor nerve terminals from predominantly fast-twitch adult lumbrical muscles are highly and selectively vulnerable to hypoxia-reperfusion injury. Fast-twitch muscle fibres rely on predominantly anaerobic respiration for rapid bursts of activity and it is known that in many respects, there is a relationship between the properties of muscle fibre sub-types and innervating α -motor neurons (see 1.4.2). For this reason it seems possible that α -motor nerve terminals, like their fast-twitch muscle fibres may also have increased provisions for sustaining ATP supply in anaerobic conditions and may also be more resistant to hypoxia/ischaemia-reperfusion injury. In previous studies however, Tombol *et al* (2002) and David *et al* (2007) have shown that α -nerve terminals from fast-twitch muscles are more vulnerable to tourniquet induced ischaemia-reperfusion injury than those from slow-twitch muscles, while others have shown a mixed response of skeletal muscle fibres (Chervu *et al.* 1989; Mars and Gregory 1991; Pedowitz *et al.* 1992; Awerbuck *et al.* 1994; Idstrom *et al.* 1990; Woitaske and McCarter 1998; Chan *et al.* 2004). The novel *ex vivo* model system developed in Chapter 2 provides the opportunity to explore possible muscle fibre type differences in α -motor nerve terminal response to hypoxia-reperfusion injury. Transversus abdominis (TA) and triangularis sterni (TS) are relatively large but very planar muscles, both composed of predominantly slow-twitch muscle fibre types (e.g., Iscoe 1998; Hodges 1999; Murray *et al.* 2008a). Consistent with the experimental design criteria discussed in Chapter 2, they can be dissected with >1cm intercostal nerve stumps and as they are thin planar muscles, maximum diffusion of O₂ across their muscle fibres will occur (Barclay 2005). Also following on from the results described in Chapter 2 where some α -motor nerve terminals were observed to fragment in response in 2H-2R to indicate the involvement of Wallerian degeneration, I will investigate the ability of the naturally occurring *Wld^f* mutation to confer protection to α -motor nerve terminals. The *Wld^f* mutation inhibits axotomy induced Wallerian degeneration, preserving the structure and function of α -motor nerve terminals for >3 days *in vivo* and in culture (Tsao *et al.* 1994; Ribchester *et al.* 1995; Gillingwater *et al.* 2002; Gillingwater *et al.* 2003; Parson *et al.* 2004). The *Wld^f* mutation has also been shown to convey neuroprotection in response to a range of pathological stimuli including CNS ischaemic-reperfusion injuries (Gillingwater *et al.* 2004). The ability of the *Wld^f* to modify the response of α -motor nerve terminals may provide valuable mechanistic insights into hypoxia-reperfusion injury.

In the following chapter, I report further morphological evidence of the vulnerability of α -motor nerve terminals to hypoxia-reperfusion injury and show significant intermuscle variability in the pathological response that is both age and fibre type dependent. I demonstrate that α -motor nerve terminal disassembly in response to hypoxia-reperfusion injury is unlikely to occur by classic Wallerian degeneration, as the *Wld^f* mutation did not convey any significant neuroprotection. I also provide preliminary evidence to show that structural pathology of α -motor nerve terminals is preceded

by functional failure to release/recycle synaptic vesicles and that the nerve terminals of the muscle spindles may be more resistant to hypoxia-reperfusion injury.

3.2 Methods

3.2.1 Animals & skeletal muscle dissection

Neuromuscular preparations were obtained from female 5-6 and 8-12 week old C57Bl/6 or 5-6 week old C57Bl/6 -*Wld^s* mice as indicated (all obtained from Harlan-Olac, UK) and were maintained as described previously in Chapter 2. In addition to the four deep lumbrical muscles (see Chapter 2 for full details), TA and TS muscles were also dissected. These planar muscles are found on the inner surface of the abdominal and thoracic wall respectively. The TS muscles attach to either side of the posterior surface of the sternum and extend to just beyond the costal cartilage of the lower 4/5 true ribs. They are respiratory muscles involved in the depression of the ribs and are supplied by 1 or 2 branches of the intercostal nerves. The TA arises either side of the vertebral column from the thoracolumbar fascia and ends on a strong connective sheet of tissue (linea alba/aponeurosis) at the midline of the anterior abdominal wall. TA is innervated by multiple branches of the lower intercostal nerves and is a postural muscle involved in compressive support and stabilisation of the abdomen. The TA and TS are almost continuous with each other as they interface behind the lower ribs (costal margin) and it is for this reason that they are always dissected together. These muscles were exposed after death by first taking the entire abdominal wall from just below the clavicle to below the iliac crests of the hips and extending to either side of the spine and pinned out in a large silicon-lined petri dish with oxygenated Krebs' solution. On the posterior surface, both TA and TS are covered in a thick layer of connective tissue (peritoneum and costal pleura respectively) but are otherwise in direct contact with Krebs' solution. On the anterior surface, TS is covered by the intercostal muscles and ribs, which cannot be removed without the risk of damage to the underlying TS. The overlying pectoral muscles were however removed along with most of the *rectus abdominis* and *external oblique* muscles that overlie the TA muscles. This leaves a window of exposed TA muscle fibres on the anterior surface around the abdominal midline just below the sternum. Those fibres located laterally and inferior to this window are covered by the thin planar internal oblique muscle, which cannot be removed until after dissection. The whole preparation was then split at the midline, along the linea alba/aponeurosis and the sternum, to create two hemi-TS/TA preparations with distal nerve stumps of ~1cm and ~1.5 respectively. From time of death to the start of experimentation, dissection of all muscles took no more than 1hr. At the end of the experimental process and after fixation, the TA window and TS were removed from surrounding tissues with fat deposits, and as much of the connective tissues/membrane removed as possible. These preparations were then mounted on to glass slides on their posterior surface after immunohistochemical processing.

3.2.2 Hypoxia-reperfusion model system and fluorescent immunohistochemistry

The hypoxia-reperfusion model system and fluorescent immunohistochemical protocol is described in detail in Chapter 2. In summary, neuromuscular preparations were maintained in 250ml of HEPES buffered Krebs' solution in a conical flask. Preparations from the right-hand side were subjected to 2hr hypoxia induced with 95%:5% N₂:CO₂ following by 2hr reperfusion induced with 95%:5% O₂:CO₂. Preparations from the left-hand side acted as controls, and were maintained for 4hr in 250ml of HEPES buffered Krebs' solution in a conical flask sparged with 95%:5% O₂:CO₂. Following experimentation, postsynaptic endplates were labelled with TRITC-conjugated α -bungarotoxin (BTX) and the preparations were fixed in MeOH for 15min at -20°C. They then underwent fluorescence immunohistochemical processing using antibodies raised against neurofilament (NF) 165kDa and synaptic vesicle 2 (SV₂) protein, visualised with Cy-3 secondary antibodies.

3.2.3 Functional Studies

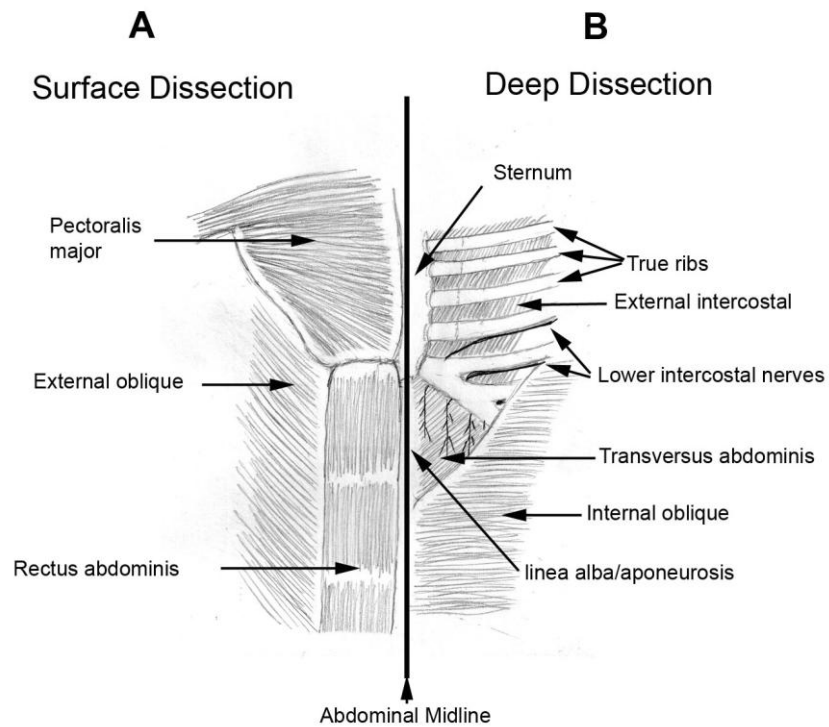
In order to assess the functional state of motor nerve terminals after 2H-2R, TA/TS muscle preparations were exposed to the fixable analogue of the styryl dye FM1-43 (FM1-43FX, 1mg/ml: Molecular Probes) for 15mins in high K⁺ Krebs' solution (102 mM Na⁺, 50 mM K⁺, 2 mM Ca²⁺, 2 mM Mg²⁺, 132 mM Cl⁻, 23.8 mM HCO₃⁻, 0.4 mM H₂PO₄²⁻, 5 mM D-glucose, 5.5mM HEPES: pH 7.2-7.4) sparged with 95%: 5% O₂:CO₂) to induce depolarisation. FM1-43FX that was not incorporated into synaptic vesicle membranes and then internalised into α -motor nerve terminals during depolarisation was washed off by a brief rinse in fresh Krebs' solution. TA and TS muscles then underwent fixation in 4% formaldehyde in PBS for 30mins, followed by a 3x10min wash in PBS. Postsynaptic endplates were labelled by incubation in alexa647-conjugated α -bungarotoxin (BTX, 5 μ g/ml in PBS: Invitrogen) for 30mins. Muscles were then washed for 2x10min and 2x15min in PBS prior to mounting on glass slides and stored as described in Chapter 2.

3.2.4 Image Capture, Quantification and Statistical analysis

Image capture, quantification and statistical analysis of α -motor nerve terminals and their endplates was carried out as previously described in Chapter 2. The pattern of muscle spindle innervation, IA/II nerve terminals and γ -neuromuscular junctions, of the lumbrical muscles was also assessed following 2H-2R and compared with controls. IA/II nerve terminals and γ -neuromuscular junctions were examined and quantified on a 'yes/no' basis. 'Yes' scores were given separately to IA/II nerve terminals and γ -neuromuscular junctions that appeared to be morphological normal while 'no' scores were given separately to IA/II nerve terminal endings and γ -neuromuscular junctions when they could not be identified. There was no occasion where either IA/II nerve terminal endings or γ -neuromuscular junctions were present ('yes') but had obvious signs of pathology. Data for each

lumbrical muscle was then pooled and the % of muscles with at least one observable IA/II nerve terminal ending or γ -neuromuscular junctions (i.e., scored 'yes') was calculated.

Anterior Abdominal/Thoracic Wall



Internal Aspect of the Thoracic Wall

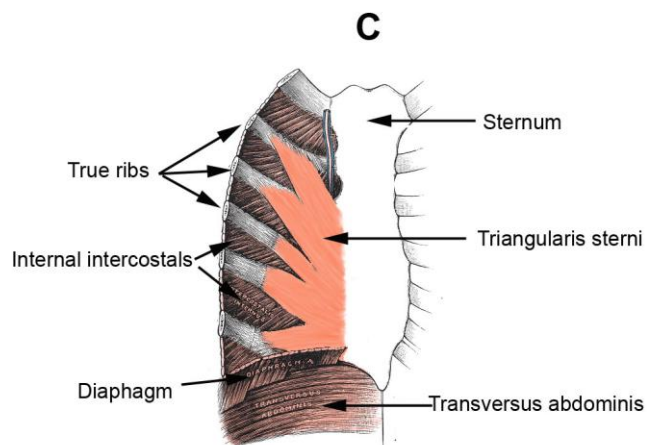


Fig 3.1 Anatomy of the abdominal/thoracic wall showing transversus abdominis (TA) and triangularis sterni (TS) muscles. (A&B) is a representation of surface (A) and deep (B) anatomy of the anterior abdominal wall based on the human. The pectoral, rectus abdominis and external oblique muscles (A) were removed to reveal a window of the transversus abdominis muscle (B) that is mostly overlaid by the internal oblique muscle that cannot be safely removed until after fixation. (C) shows the internal aspect of the human thoracic wall with the triangularis sterni muscle pseudo-coloured. As this muscle is overlaid by the ribs and the intercostal muscles it cannot be fully exposed on the anterior surface until after fixation. Both TA and TS muscles are innervated by the intercostal nerves that exit the spinal cord and track along the lower aspects of the ribs. For the 2H-2R protocol, preparations were split up the abdominal midline, i.e., along the connective tissue of the linea alba/ aponeurosis and up the centre of the sternum to produce two TA/TS muscle preparations. Following the 2H-2R and fixation, the TA window was removed (that always corresponded to the area of muscle innervated by the first 3/4 branches of its intercostal nerve supply) and TS was dissected free of its overlying tissue. Image (C) adapted from the bartleby.com edition of Gray's *Anatomy of the Human Body*

3.3 Results

3.3.1 α -motor nerve terminals from slow-twitch muscles, TA and TS, are slower to respond/more resistant to hypoxia-reperfusion injury than fast-twitch lumbrical muscles.

The effects of 2hr hypoxia followed by 2hr reperfusion (2H-2R) on the morphology of pre- and post-synaptic components of the mouse neuromuscular junction in the TA and TS muscles, aged 8-12wks, from the right-hand side of the abdominal and thoracic wall respectively were compared with those muscles from the left hand side kept under control conditions. 2H-2R did not cause any identifiable changes to the integrity/morphology of post-synaptic endplates or muscle fibres compared with control preparations in either TA (Fig 3.2) or TS (Fig 3.3) muscles. Both TA and TS muscles showed a noticeable loss of NF/SV₂ immunoreactivity in response to 2H-2R, with $58.9 \pm 11.4\%$ and $27.7 \pm 7.8\%$ of endplates appearing vacant in TA ($N=4$, $n=4$ (1753)) and TS ($N=4$, $n=4$ (1765)) muscles respectively. This contrasted with control values where 100% and $99.8 \pm 0.2\%$ of nerve terminals fully opposed their postsynaptic endplates in TA ($N=3$, $n=3$ (2123)) and TS ($N=4$, $n=4$ (2069)) muscles (Fig 3.4). Intermuscle comparison showed that the difference between the percentage of vacant endplates from TA and TS muscles was not significant (Fig 3.4C). When data from TA and TS was statistically compared to that of 4 deep lumbricals (see Chapter 2 for details), the decreased number of vacant endplates in TA muscle compared to that of the lumbrical muscles was also not statistically different. The difference between the number of vacant endplates in TS and lumbrical muscle however was statistically significant ($P=0.0034$). This would indicate that TS and to a lesser extent TA, both predominantly composed of slow-twitch fibres, are either more resistant or slower to respond to hypoxia-reperfusion injury than predominantly fast-twitch lumbrical muscle.

In line with results obtained from lumbrical muscles, there was a small population of endplates in TA and TS muscles that were observed to be only partially opposed by presynaptic NF/SV₂ immunoreactivity in response to 2H-2R ($4.8 \pm 0.8\%$ and $3.3 \pm 1.2\%$ of TA and TS nerve terminals respectively) which were only rarely observed in control preparations (0% and $0.1 \pm 0.1\%$ in TA and TS respectively: Fig3.4A&B). These partial α -motor nerve terminals also had a similar range of morphological characteristics as previously described in lumbrical muscles, where some α -motor nerve terminals appeared to be undergoing Wallerian-like fragmentation while others appeared to be withdrawal-like processes of disassembly (Fig3.2&3, E&F). It was noted that there was an increased frequency of partially occupied endplates with $4.8 \pm 0.8\%$ and $3.3 \pm 1.2\%$ of TA and TS nerve terminals respectively observed with a partial morphology compared to $1.4 \pm 0.3\%$ observed in lumbrical muscles (Fig3.4D). Intermuscular comparison showed that the percentage of partially occupied endplates in TA was significantly ($P=0.0048$) more than that in lumbrical muscles. TS also showed an

increase in the number of partially occupied endplates but this did not reach significance. This would suggest that α -motor nerve terminals from TA muscles and to a lesser extent TS muscles are slower to undergo disassembly than lumbrical muscles following hypoxia-reperfusion injury. This is most likely due to the generally larger and more complex morphology α -motor nerve terminal arborisation in slow-twitch muscles observed here and described by others (Wood and Slater 1997; Bewick *et al.* 2004).

3.3.2 Reduced vesicle release/recycling in α -motor nerve terminals from TA and TS following hypoxia-reperfusion injury

The immunohistochemical data collected here and in Chapter 2 suggests disruption of proteins related to synaptic vesicles, (i.e., SV₂) and would therefore indicate disturbance of synaptic vesicle release/recycling. As TA and TS are thin, planar muscles they are highly suited to functional studies with the vital dye FM1-43FX as larger thicker muscles, like the lumbricals, can suffer from poor penetration of the dye. FM1-43FX can be used to assess synaptic release/recycling as the lipophilic tail regions of the dye become weakly embedded into the exposed membranes of vesicles as they bind with the presynaptic membrane. The dye then becomes internalised into nerve terminals during endocytosis while non-internalised dye can then be washed off (Betz *et al.* 1992; Rizzoli *et al.* 2003; Adalbert *et al.* 2005). Rather than electrically stimulating the nerve stump, depolarisation was produced with a high (50mM) K⁺ stimulus to avoid the potential complications of axonal conduction block caused by 2H-2R. Neurons typically have a resting membrane potential ranging from approximately -65mV to -90mV, which arises largely due to the tendency of K⁺ to leak out the cell down its concentration gradient. This concentration gradient is in turn caused by the Na⁺/K⁺ATPase exchanger that generates a high concentration of K⁺ and a low concentration of Na⁺ in the cytosol (see e.g., Kandel and Schwartz 1985; Longstaff 2000; Lodish *et al.* 2008). Neurons need to be depolarised to 40-70mV to trigger exocytosis (Kandel and Schwartz 1985) and during an action potential depolarisation this is achieved by the inward flow of Na⁺ via voltage gated ion channels (see e.g., Kandel and Schwartz 1985; Longstaff 2000; Lodish *et al.* 2008). Application of 50mM K⁺ stimulus however acts simply to reduce the K⁺ concentration gradient across the cell membrane and thereby causing the resting membrane potential to be approximately 40mV (i.e., depolarised: Katz 1966). By depolarising with high K⁺, exocytosis (and therefore endocytosis (Zefirov *et al.* 2006)) is maintained by a constant influx of Ca²⁺ (via voltage gated Ca²⁺) adjacent to the active zones in the nerve terminals.

α -motor nerve terminals were depolarised in TA ($n=2$) and TS ($n=2$) muscles following 2H-2R by transferring them into a high K⁺ Krebs' solution to load synaptic terminals with the dye (Adalbert *et al.* 2005: Fig 3.5). No nerve terminals from 2H-2R treated muscles with clear FM1-43FX fluorescence were identified. By contrast, in control preparations nearly all terminals examined showed clear FM1-43FX fluorescence. This suggests that 2H-2R treated terminals have a significantly reduced ability to

release/recycle synaptic vesicles. Compared with the immunohistochemical data where TA and TS showed $57.9 \pm 11.4\%$ and $27.7 \pm 7.8\%$ loss of NF/SV₂ immunoreactivity following 2H-2R, this data suggests that functional disruption may occur prior to the morphological signs of disassembly.

3.3.3 Age dependent increase in the vulnerability of α -motor nerve terminals from TA and TS muscles to hypoxia-reperfusion injury.

I wished to determine whether the *Wld^s* mutation could protect α -motor nerve terminals from 2H-2R, but *Wld^s* mediated neuroprotection is age-dependent, with electrophysiological signs of weakening beginning at 10wks of age (Derek Thompson, University of Edinburgh, personal communication) and protection being lost by 16wks of age (Gillingwater *et al.* 2002). To fully assess the ability of the *Wld^s* mutation to convey protection against 2H-2R, it was necessary to use juvenile animals (elected age, 5-6wks) but prior to this, it was important to determine if similar responses to 2H-2R occurred in wild-type juvenile animals. Using lumbrical, TA and TS muscles, I examined α -motor nerve terminal pathology following 2H-2R in tissue from juvenile mice aged 5-6wks. In line with previous results 2H-2R did not cause any major perturbations to postsynaptic endplate or muscle fibre morphology/integrity in lumbrical (Fig 3.6), TA (Fig 3.7) or TS (Fig 3.8) muscles. In response to 2H-2R, $84.8 \pm 4.2\%$ of postsynaptic endplates in lumbrical muscles aged 5-6wks had no opposing NF/SV₂ immunoreactivity ($N=4$, $n=16$ (3697)) which was significantly different ($P<0.0001$) from only $1.1 \pm 0.8\%$ of vacant endplates observed in control preparations ($N=4$, $n=16$ (3493): Fig 3.9A). As observed in lumbrical muscles aged 8-12wks (Fig 2.7B), the 4th deep lumbrical from 5-6wk old mice showed a decreased response to 2H-2R (Fig 3.9D), which was significant ($P=0.029$) when compared to the percentage of vacant endplates found in the 2nd deep lumbrical muscle. This would again indicate subtle intra-lumbrical differences in the response to 2H-2R however, the actual significance of this should be treated with caution due to the high degree of observed variance in both 5-6wk and 8-12wk old 4th lumbrical muscles and the lower number of repeat experiments from 5-6wk old animals. The numbers of partial α -motor nerve terminals was also similar in 5-6wk old ($2.2 \pm 0.6\%$) lumbrical muscles, and again showed signs of fragmented and withdrawing morphologies (Fig 3.6E&F). Overall, there was no significant difference in the response of lumbrical muscles from mice aged 5-6wks compared to mice aged 8-12wks (Fig 3.9E) to 2H-2R. This contrasts with the response of TA and TS muscles from animals aged 5-6wks which showed a significantly ($P=0.0286$) increased response to 2H-2R compared with TA and TS muscles from animals aged 8-12wks (Fig 3.9E). In TA and TS muscles aged 5-6wks, $99.4 \pm 0.2\%$ ($N=4$, $n=4$ (2104): Fig 3.9B) and $97.3 \pm 1.4\%$ ($N=4$, $n=4$ (2220): Fig 3.9C) of endplates appeared with no opposing NF/SV₂ immunoreactivity which directly contrasts with control muscles where 0% ($N=3$, $n=3$ (1381)) and $0.3 \pm 0.3\%$ ($N=3$, $n=3$ (1912)) of endplates appeared vacant in TA and TS respectively. Only a very small population of α -motor nerve terminals could be observed with either a partial (TA: $0.4 \pm 0.1\%$, TS: $1.3 \pm 0.7\%$) or full (TA: $0.2 \pm 0.2\%$, TS: $1.4 \pm 0.8\%$) morphology following 2H-2R. The slightly larger number of partial

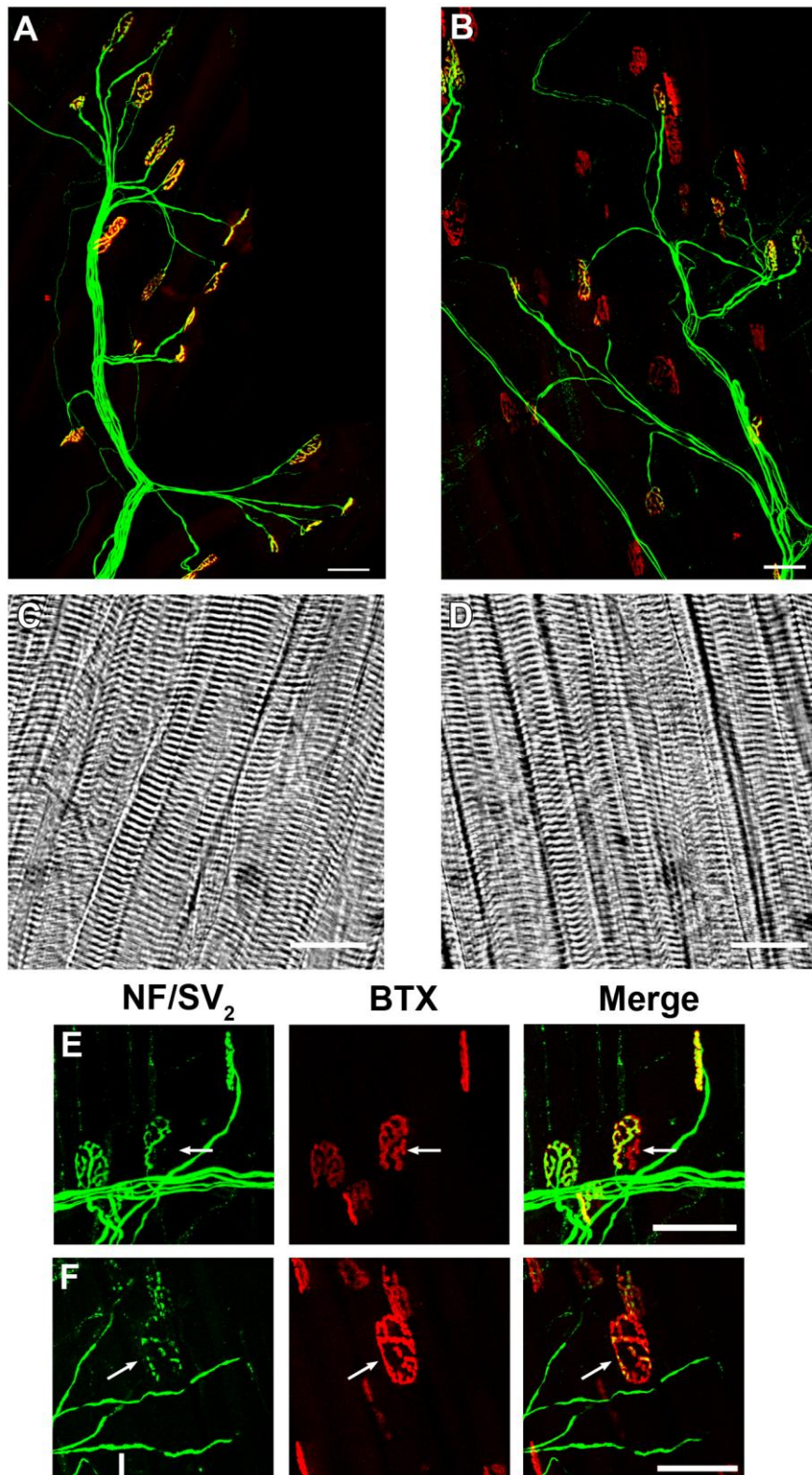


Fig 3.2 2H-2R causes selective α -motor nerve terminal disassembly in TA muscles (8-12wks). Reconstructed confocal projections of part of a mouse TA muscle aged 8-12wks after being maintained in control conditions (**A**) and following 2H-2R (**B**). While postsynaptic endplates (visualised with BTX: RED) appear unchanged, there is widespread loss of presynaptic NF/SV₂ immunoreactivity (GREEN) following 2H-2R. 2H-2R does not appear to affect muscle fibres as phase-contrast micrographs show uniform muscle fibres with clear striations in both control (**C**) and 2H-2R treated TA muscles (**D**). Confocal projections of a small population of endplates following 2H-2R appear partially occupied by presynaptic NF/SV₂ immunoreactivity (**E&F**). In (**E**) there is asynchronous loss of NF/SV₂ at one endplate (arrow) which is indicative of a withdrawal-like mechanism. In contrast (**F**) shows an endplate (arrow) with fragmented NF/SV₂ immunoreactivity that is more consistent with a Wallerian-like mechanism. Scale bars: 50 μ m

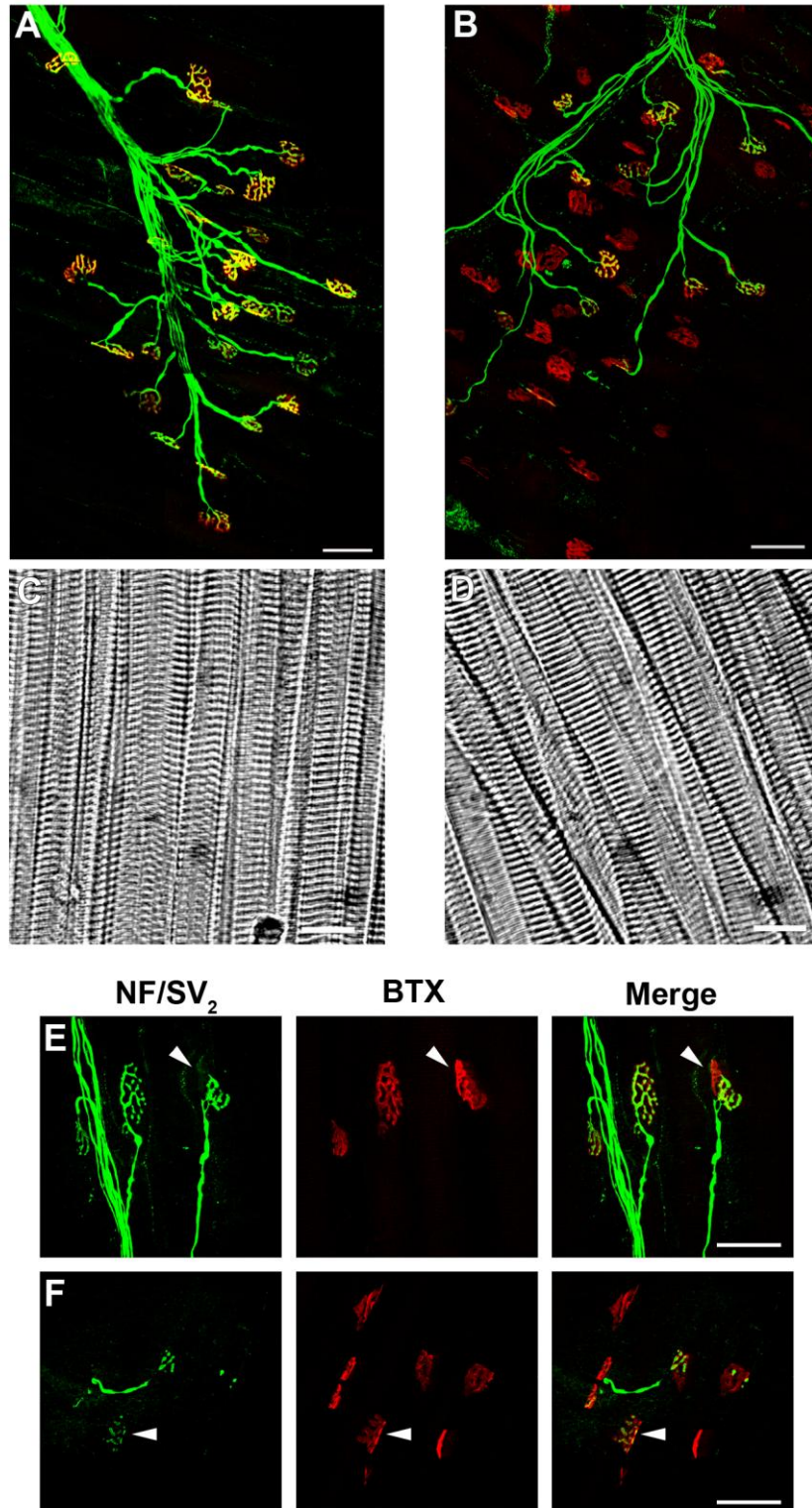


Fig 3.3 2H-2R causes selective α -motor nerve terminal disassembly in TS muscles (8-12wks). Reconstructed confocal projections of part of a mouse TS muscle aged 8-12wks after being maintained in control conditions (**A**) and following 2H-2R (**B**). While postsynaptic endplates (visualised with BTX: RED) appear unchanged, there is widespread loss of presynaptic NF/SV₂ immunoreactivity (GREEN) following 2H-2R. 2H-2R does not appear to affect muscle fibres as phase-contrast micrographs show uniform muscle fibres with clear striations in both control (**C**) and 2H-2R treated TS muscles (**D**). Confocal projections of a small population of endplates following 2H-2R appear partially occupied by presynaptic NF/SV₂ immunoreactivity (**E&F**). In (**E**) there is asynchronous loss of NF/SV₂ at one endplate (arrow) which is indicative of a withdrawal-like mechanism. In contrast (**F**) shows an endplate (arrow) with fragmented NF/SV₂ immunoreactivity that is more consistent with a Wallerian-like mechanism. Scale bars: 50 μ m

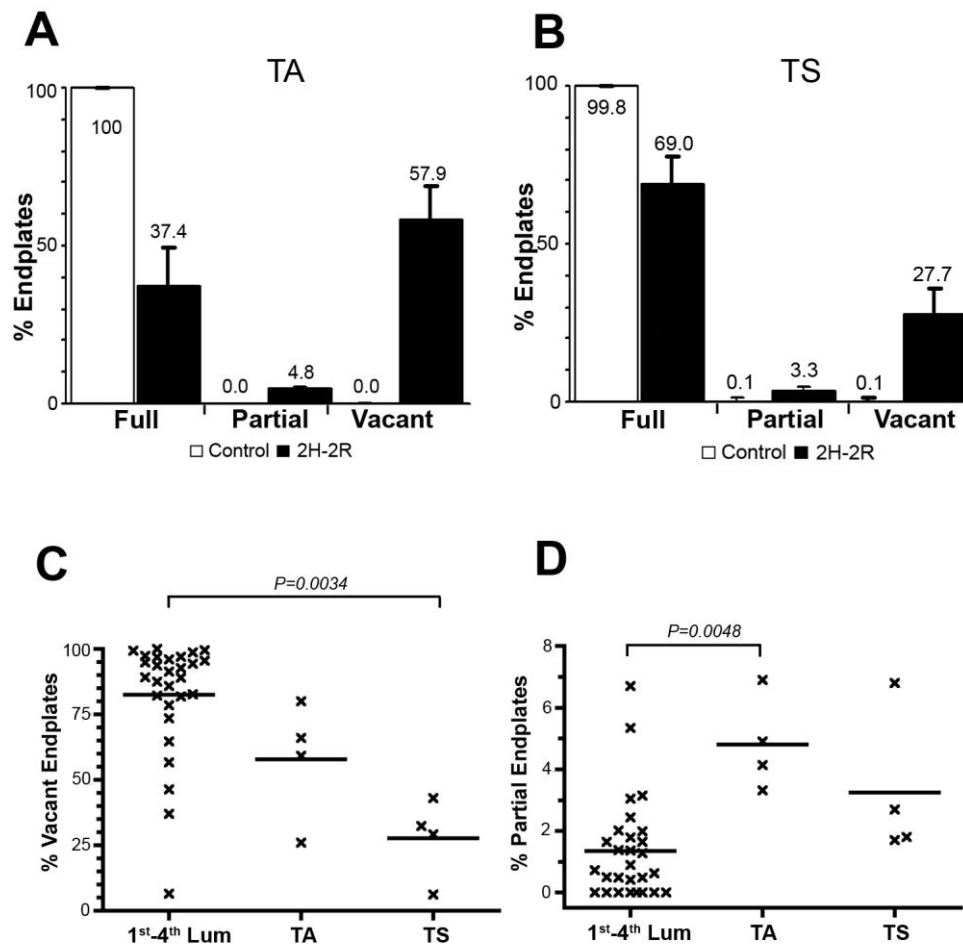


Fig 3.4 Quantification of α -motor nerve terminal response to hypoxia-reperfusion injury shows there are significant inter-muscle differences in 8-12wk mice that may be related to fibre type. Bar charts (%mean \pm SE) showing the categorical quantification of α -motor nerve terminal status (i.e., fully-occupied, partially-occupied or vacant) that oppose postsynaptic endplates from TA (**A**) and TS (**B**) muscles where 2H-2R treated muscles (black bars) can be compared to controls (white bars). In (**C**), a scatter plot (%mean indicated by line) showing inter-muscle differences in the number of endplates identified with no NF/SV₂ immunoreactivity, i.e., vacant, following 2H-2R injury (data from all 4 deep of lumbrical (lum) muscles from chapter 2). Statistical analysis showed there is no significant difference between TA and TS muscles that are both composed of predominantly slow type fibres but in comparison with lumbrical muscles, composed of predominantly fast type fibres, there was a significant difference between the %number of vacant endplates observed in the lumbrical muscles and the TS muscle. In (**D**), a scatter plot shows some inter-muscle differences in the percentage of endplates observed with a partial nerve terminal morphology. Statistical analysis showed there is a significant difference between the %number of partial endplates observed in the lumbrical muscles and TA muscles.

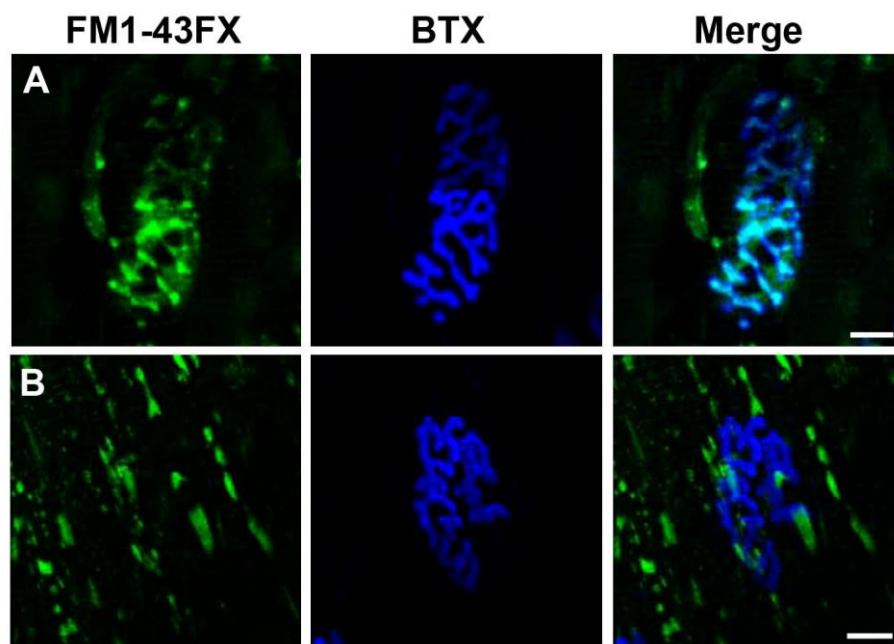


Fig 3.5 Loss of synaptic vesicle release/recycling following 2H-2R. In control conditions (**A**), confocal projections show postsynaptic endplates (visualised with BTX: BLUE) of TS muscles are opposed by clear presynaptic FM1-43FX (GREEN) that has become incorporated into synaptic vesicles as they are released/recycled following depolarisation with high K^+ . Following 2H-2R (**B**), there is no clear presynaptic FM1-43FX staining opposing endplates suggesting a failure in synaptic vesicle release/recycling following depolarisation with high K^+ . Scale bar 10 μ m

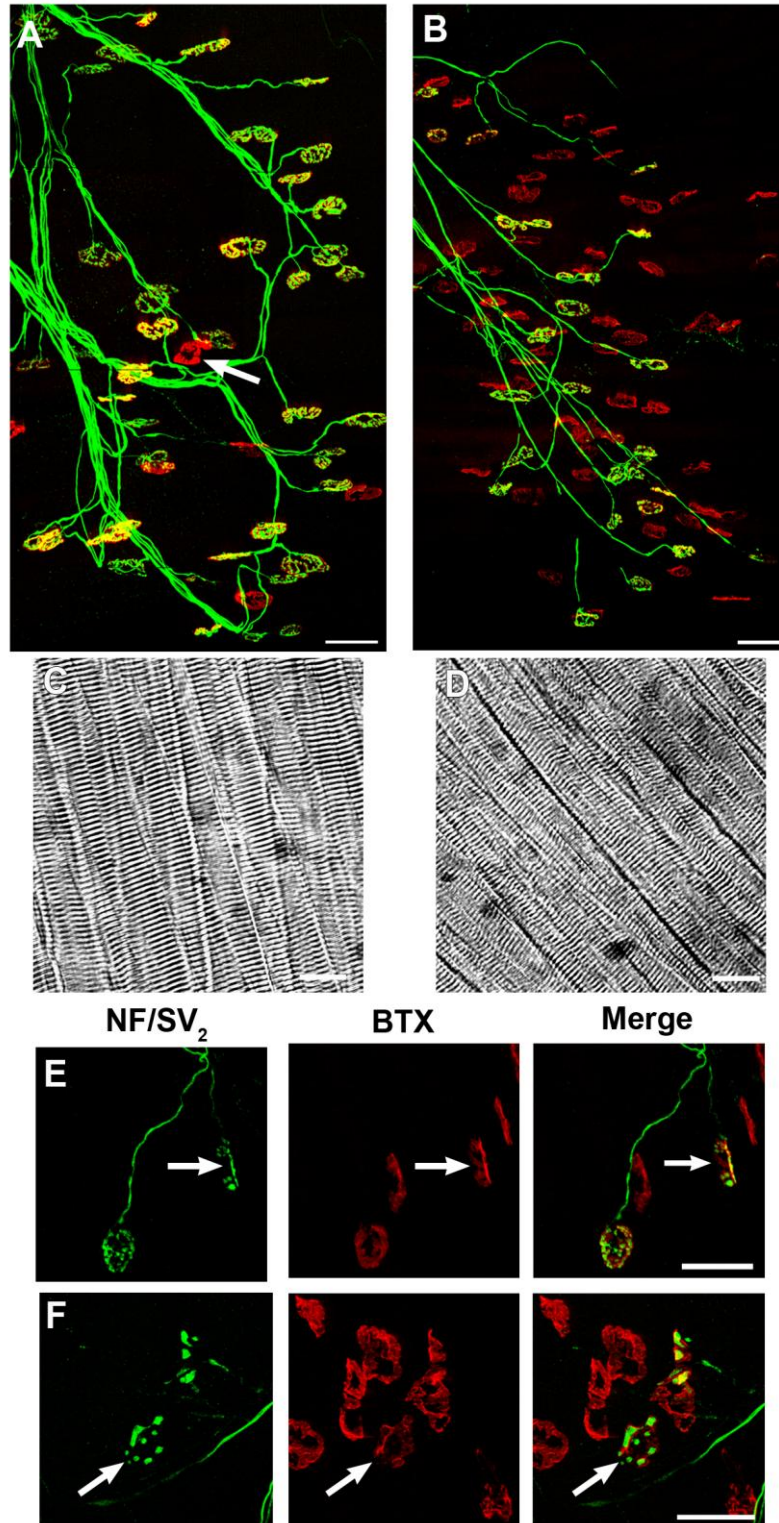


Fig 3.6 2H-2R causes selective α -motor nerve terminal disassembly in lumbrical muscles from mice aged 5-6wks. Reconstructed confocal projections of part of a mouse lumbrical muscle aged 5-6wks after being maintained in control conditions (**A**) and following 2H-2R (**B**). In controls (**A**) most postsynaptic endplates (visualised with BTX: RED) are fully opposed by presynaptic NF/SV₂ immunoreactivity (GREEN) however a small cluster of endplates (e.g., arrow) can be observed to have no opposing NF/SV₂ immunoreactivity. This is most likely the result of naturally occurring pathophysiological processes. Following 2H-2R, postsynaptic endplates appear unchanged but there is widespread loss of presynaptic NF/SV₂ immunoreactivity. 2H-2R does not appear to affect muscle fibres as phase-contrast micrographs show uniform muscle fibres with clear striations in both control (**C**) and 2H-2R treated lumbrical muscles (**D**). Confocal projections of a small population of endplates following 2H-2R appear partially occupied by presynaptic NF/SV₂ immunoreactivity (**E&F**). In (**E**) there is asynchronous loss of NF/SV₂ at one endplate (arrow) which is indicative of a withdrawal-like mechanism. In contrast (**F**) shows an endplate (arrow) with fragmented NF/SV₂ immunoreactivity that is more consistent with a Wallerian-like mechanism. Scale bars: 50 μ m

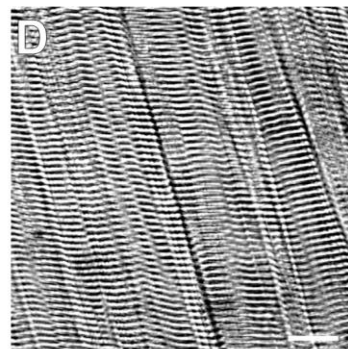
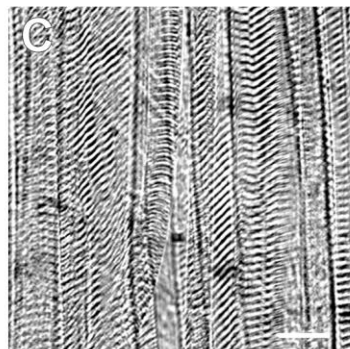
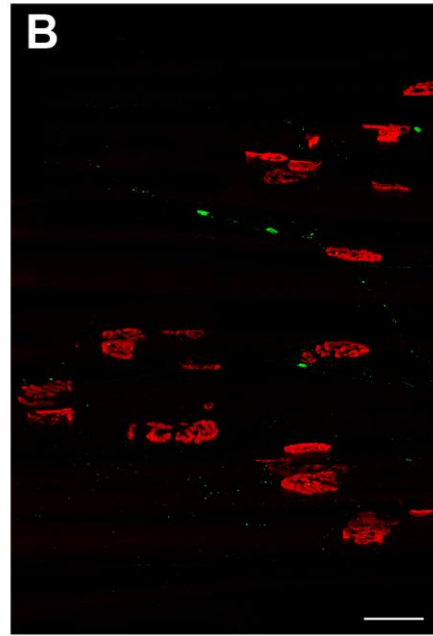
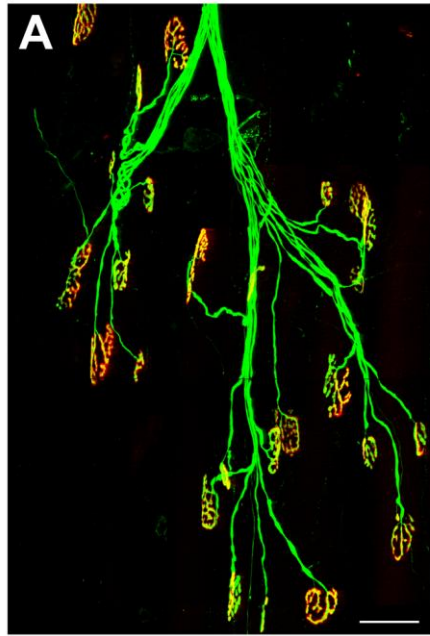


Fig 3.7 2H-2R causes selective α -motor nerve terminal disassembly in TA muscles from mice aged 5-6wks. Reconstructed confocal projections of part of a mouse TA muscle aged 5-6wks after being maintained in control conditions (**A**) and following 2H-2R (**B**). While postsynaptic endplates (visualised with BTX: RED) appear unchanged, there is widespread loss of presynaptic NF/SV₂ immunoreactivity (GREEN) following 2H-2R. 2H-2R does not appear to affect muscle fibres as phase-contrast micrographs show uniform muscle fibres with clear striations in both control (**C**) and 2H-2R treated TA muscles (**D**). In contrast to other muscles there were almost no occurrences of endplates partially occupied by presynaptic NF/SV₂ immunoreactivity (see Fig 2.9B). Scale bars: 50 μ m.

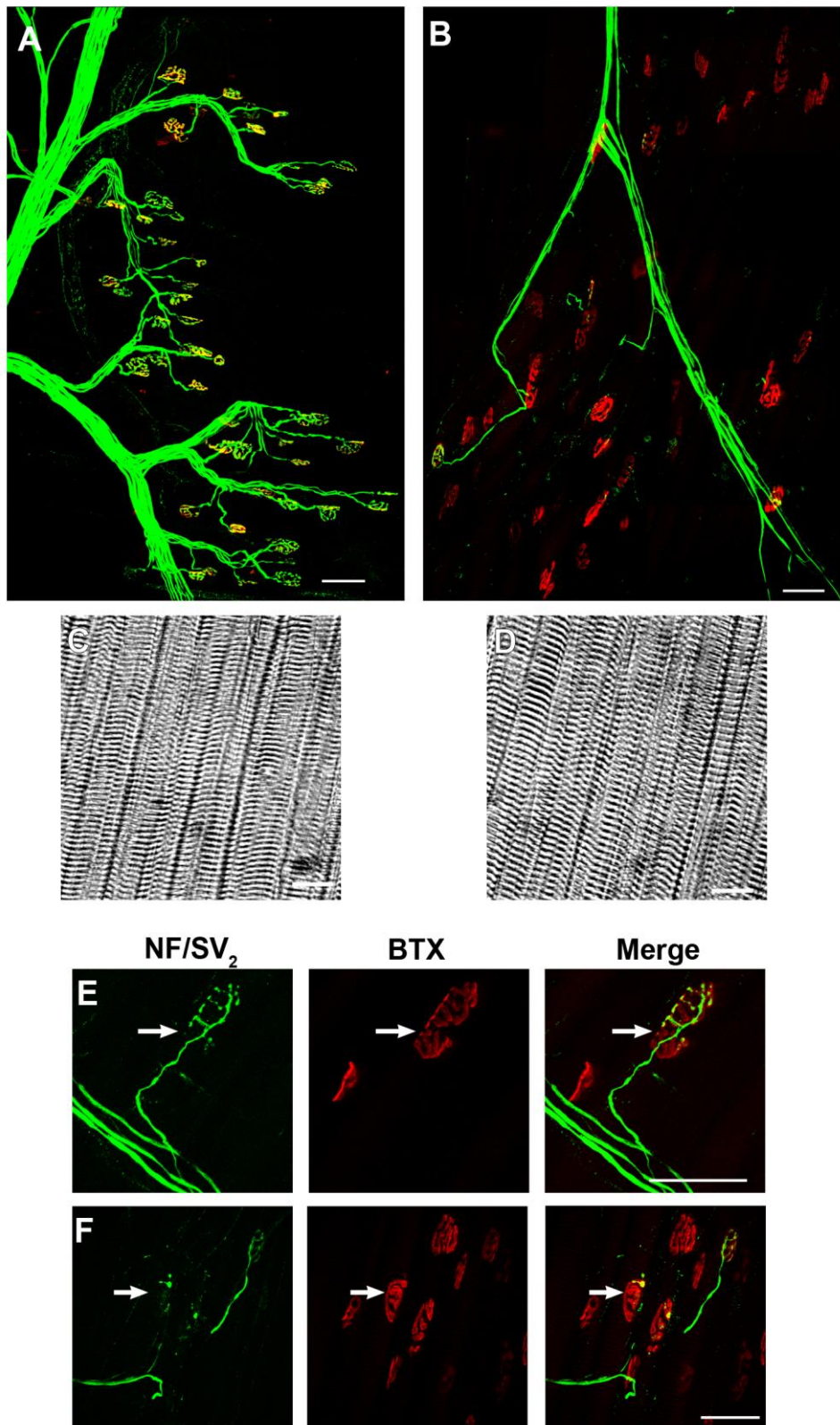


Fig 3.8 2H-2R causes selective α -motor nerve terminal disassembly in TS muscles from mice aged 5-6wks. Reconstructed confocal projections of part of a mouse TS muscle aged 5-6wks after being maintained in control conditions (**A**) and following 2H-2R (**B**). While postsynaptic endplates (visualised with BTX: RED) appear unchanged, there is widespread loss of presynaptic NF/SV₂ immunoreactivity (GREEN) following 2H-2R. 2H-2R does not appear to affect muscle fibres as phase-contrast micrographs show uniform muscle fibres with clear striations in both control (**C**) and 2H-2R treated TS muscles (**D**). Confocal projections of a small population of endplates following 2H-2R appear partially occupied by presynaptic NF/SV₂ immunoreactivity (**E&F**). In (**E**) there is asynchronous loss of NF/SV₂ at one endplate (arrow) which is indicative of a withdrawal-like mechanism. In contrast (**F**) shows an endplate (arrow) with fragmented NF/SV₂ immunoreactivity that is more consistent with a Wallerian-like mechanism. Scale bars: 50 μ m

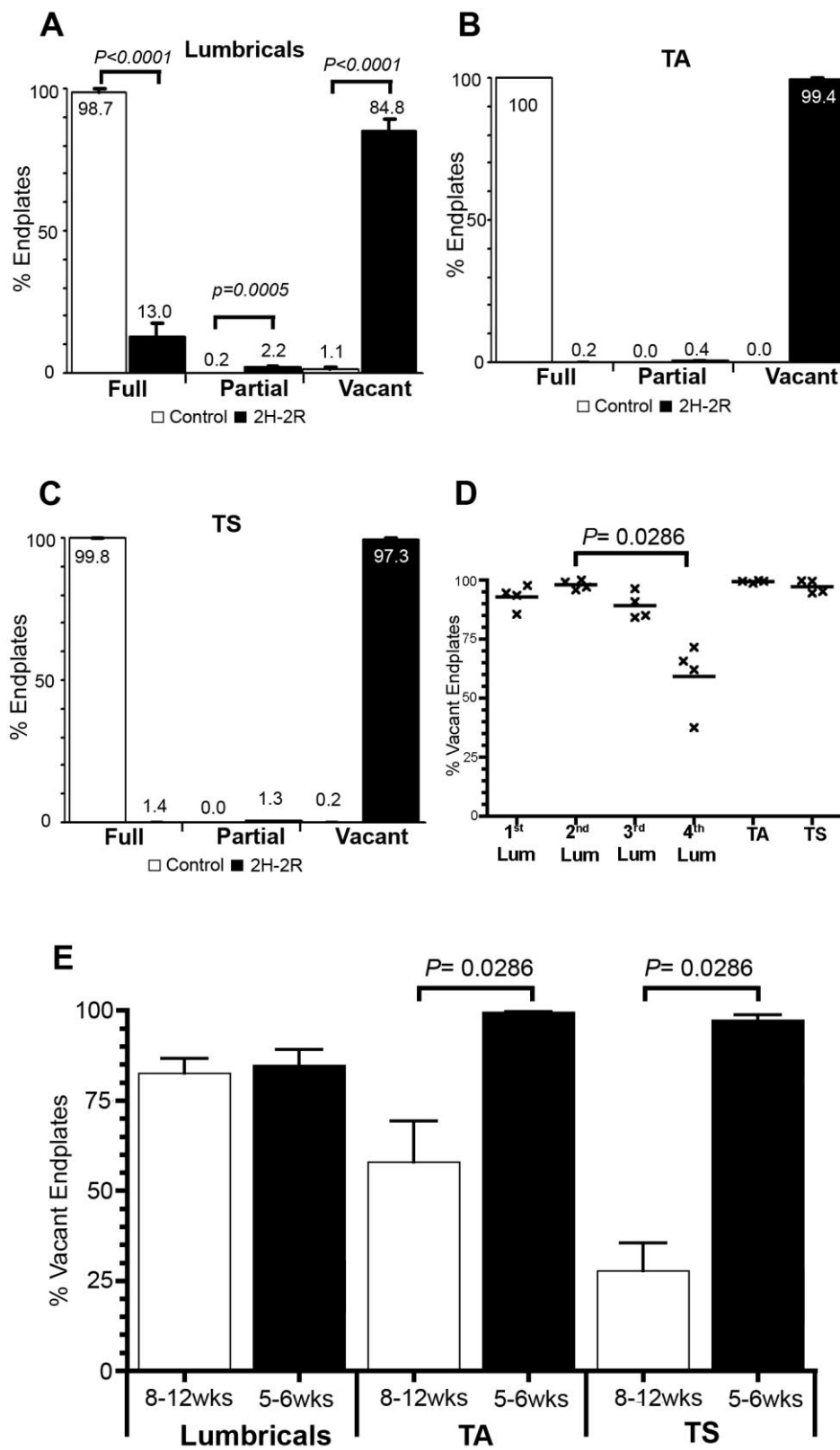


Fig 3.9 Quantification of α -motor nerve terminal response to hypoxia-reperfusion injury shows there are significant age related differences between muscles. Bar charts (%mean \pm SE) showing the categorical quantification of α -motor nerve terminal status (i.e., fully-occupied, partially-occupied or vacant) that oppose postsynaptic endplates in lumbrical (**A**), TA (**B**), and TS (**C**) muscles from mice aged 5-6wks, where 2H-2R treated muscles (black bars) can be compared to controls (white bars). In (**D**), a scatter plot (%mean indicated by line) showing inter-muscle differences in the number of endplates identified with no NF/SV₂ immunoreactivity, i.e., vacant, following 2H-2R injury). Similar to the results described in Chapter 2 in 8-12wk old mice, there were some intra-lumbrical differences in 5-6wk old mice, with the 4th lumbrical (Lum) showing a decreased response that was statistically significant when compared to the 2nd lumbrical muscle. In contrast to the results obtained in 8-12wk old animals, in 5-6wk old animals there were no statistically significant differences in the percentage of vacant endplates in response 2H-2R between lumbrical, TA and TS muscles. In (**E**) a bar chart compares the percentage of vacant endplates from all three muscles following 2H-2R in animals aged 5-6wks (black bars) and 8-12wks (white bars). In both TA and TS muscles there is statistically significant increase in the percentage of vacant endplates in 5-6wk old animals compared to 8-12wk old animals. The percentage of vacant endplates in the 4 lumbrical muscles appears unchanged in the two age groups.

morphologies in TS muscle showed a similar range of morphologies as described previously with aspects of fragmentation and withdrawal. Overall, these results show age and muscle type-dependent changes in α -motor nerve terminal vulnerability to hypoxia-reperfusion injury, with fast-twitch lumbrical muscles being unaffected by age but slow-twitch muscles, TA and TS, being more vulnerable to 2H-2R at younger ages.

3.3.4 The *Wld^s* mutation does not convey protection to α -motor nerve terminals against hypoxia-reperfusion injury

Next, I investigated if mice carrying the *Wld^s* mutation could protect α -motor nerve terminals from hypoxia-reperfusion injury. The fragmented partial morphologies observed in response to 2H-2R in previous experiments implicated Wallerian-like degenerative pathways and the *Wld^s* mutation significantly delays Wallerian degeneration in the CNS and PNS (Lunn *et al.* 1989; Ribchester *et al.* 1995; Gillingwater *et al.* 2003; Gillingwater *et al.* 2006a; Gillingwater *et al.* 2006b; Wishart *et al.* 2007) and has been shown to be protective in ischaemia-reperfusion injuries in the CNS (Gillingwater *et al.* 2004). Compared to wild type animals of 5-6wks of age described above, the *Wld^s* mutation conveyed no significant (Fig 3.13E) neuroprotective effect on α -motor nerve terminals in lumbrical (Fig 3.10), TA (Fig 3.11) or TS (Fig 3.12) muscles against 2H-2R. For *Wld^s* lumbrical muscles values for vacant endplates with no apparent NF/SV₂ immunoreactivity were: $82.4 \pm 2.9.3\%$ ($N=6$, $n=24$ (5468): Fig 3.13A), TA $98.1 \pm 0.7\%$ ($N=5$, $n=5$ (2568): Fig 3.13B) TS $96.6 \pm 1.2\%$ ($N=5$, $n=5$ (2290): Fig 3.13C). This contrasts with control preparations from *Wld^s* mice where only $0.5 \pm 0.1\%$ and $1.0 \pm 0.4\%$ of endplates were observed to have either a partial or vacant morphology in lumbrical muscles ($N=3$, $n=12$ (2718)) contrasting with no partial or vacant endplates observed in control *Wld^s* TA ($N=3$: $n=3$ (1934)) or TS ($N=3$: $n=3$ (1894)) muscles. In line with previous experiments, there were subtle but non-significant intra-lumbrical differences (Fig 3.13D) in percentages and variance of vacant endplates. Curiously, this affected the 1st deep lumbrical muscles as well as the 4th deep lumbrical muscles described previously. There were also no major differences in the percentage of partially occupied endplates in *Wld^s* mice in response to 2H-2R (values: Lumbrical $1.5 \pm 0.3\%$, TA 1.2 ± 0.3 and TS $1.8 \pm 0.6\%$) and a similar range of fragmenting and withdrawing morphologies were observed (Figs 3.10-12). In summary, the failure of *Wld^s* mutation to protect α -motor nerve terminals against 2H-2R injury or alter the apparent mechanism(s) of α -motor nerve terminal disassembly suggests that nerve terminal disassembly is occurring via mechanisms distinct from classic Wallerian degeneration.

3.3.5 γ -motor and IA/II sensory nerve terminals may be more resistant to hypoxia-reperfusion injury.

During the course of experiments on lumbrical muscle described here and in Chapter 2, it was noted that muscle spindles (between 1 and 2 per muscle) often appeared to be preserved following 2H-2R in 8-12wks (Fig 3.14) 5-6wks (Fig 3.15) and 5-6wk *Wld^f* lumbrical muscles (Fig 3.16). The muscle spindle is the name used to collectively describes the specialised sensory IA/II afferent and motor γ -efferent innervations, (as well as the capsule that surrounds them and the intrafusal muscle fibres) that provide sensory feedback to aid proprioception and antagonise/co-ordinate skeletal muscle contraction. Identified here with NF/SV₂ immunoreactivity, sensory IA/II afferent nerve terminals typically form evenly spaced and regular spiral or half-ring annulospiral endings that are even in diameter around the equatorial/ subequatorial regions of the intrafusal fibres as described by (Sherrington 1893; Boyd 1962; Barker 1974: Figs3.14-16). Alternatively and much less common (Barker and Ip 1960), rather than observing evenly spaced spirals some endings appeared to be composed of scattered varicosities which would be consistent with II secondary 'flower spray' endings originally described by (Ruffini 1898:Fig 3.16B). The γ -motor innervations are found towards the polar regions of the intrafusal muscle fibres and terminate in small γ -neuromuscular junctions that form either singular/small groups of varicosities at the distal regions of their muscle fibres or form numerous 'trails' of varicosities extending away from the IA/II endings. Unlike IA/II endings, these γ - nerve terminals are apposed by small endplates that have postsynaptic AChRs that can be identified by BTX. The relative intensity of BTX staining could be highly variable with the appearance of these endplates ranging from intense bright plaques to unidentifiable against background fluorescence. This high degree of variability in both the IA/II endings and the γ -neuromuscular junctions has been previously reported and in many cases represent differences in the innervation patterns between the bag and chain intrafusal muscle fibres that make-up the muscle spindle (e.g. Sherrington 1893; Ruffini 1898; Boyd 1962; Barker *et al.* 1970; Barker 1974; Boyd and Gladden 1985). For the purposes of this study however, innervation was simply characterised as either being IA/II endings or γ -neuromuscular junctions. Overall, analysis of the possible response and extent of response(s) of both IA/II endings and γ -neuromuscular junctions to 2H-2R was hindered by difficulties in identifying these nerve terminals and determining pathology. The thick collagen-containing capsule that surrounds the muscle spindle can inhibit penetration of antibodies (Dow *et al.* 1980) creating difficulties in determining if lack of immunoreactivity was due to hypoxia-reperfusion injury. The lack of postsynaptic BTX apposing IA/II endings and the variable BTX staining apposing γ -motor nerve terminals also made identification of possible 'partial' as well as 'vacant' nerve terminal morphologies very difficult. It must however be noted that on no occasion during the course of this study was any obvious pathology noted in IA/II or γ - nerve endings (e.g. breaks/fragmentation/swellings/atrophy of NF/SV₂ immunoreactivity) either following 2H-2R or in

controls. Rather, IA/II and γ -neuromuscular junctions either had completely normal morphology or were absent. To try and determine if 2H-2R was affecting muscle spindle innervation, the percentage of muscles observed to have IA/II endings and γ -neuromuscular junctions (each assessed independently) following 2H-2R was compared to controls (Fig3.17). In response to 2H-2R, a large decrease in the number of IA/II endings was observed in 5-6wk old animals, where only 56% of lumbrical muscles ($N=4$, $n=16$) could be observed to have IA/II endings followed 2H-2R compared to 100% of muscles from 5-6wk old controls ($N=4$, $n=16$). In 8-12wk old animals and 5-6wk old *Wld^f* animals, 75% ($N=7$, $n=28$) and 78% ($N=7$, $n=28$) of muscles had IA/II nerve terminal ending respectively and 86% and 93% of muscles had γ -neuromuscular junctions respectively. When these values are compared to control values however this is unlikely to reflect any major response to 2H-2R: in 8-12wk old animals and 5-6wk old *Wld^f* animals, 67% ($N=6$, $n=24$) and 79% ($N=7$, $n=28$) of muscles had IA/II nerve terminal ending respectively and 100% and 89% of muscles had γ -neuromuscular junctions respectively. The less than maximal presence of IA/II endings and γ -neuromuscular junction in control 8-12wk and 5-6wk *Wld^f* animals most likely reflects poor penetration of NF/SV₂ antibodies into the capsule and the difficulty in identifying these terminals and therefore, the results obtained from 5-6wk old animals needs to be treated with caution. When these figures are compared to the number of α -motor nerve terminals retaining a full morphology following 2H-2R (between 13% and 16.1% from all lumbrical muscles) however, this data would indicate that IA/II ending and γ -nerve terminals that innervate intrafusal muscle fibres maybe more resistant to hypoxia-reperfusion injury than α -motor nerve terminals that innervate extrafusal muscle fibres. This would support the general finding of selective vulnerability/resistance of nerve terminals to hypoxia-reperfusion injury and indicates that vulnerability may not only determined by age and muscle type differences but also neuron/nerve terminal type. This however remains a preliminary finding as overall, the results obtained here are difficult to interpret due to the staining caveats and do not lend themselves to any sure conclusions regarding the extent to which IA/II ending and γ -nerve terminals may be vulnerable to hypoxia-reperfusion injury or the ability of the *Wld^f* to convey protection.

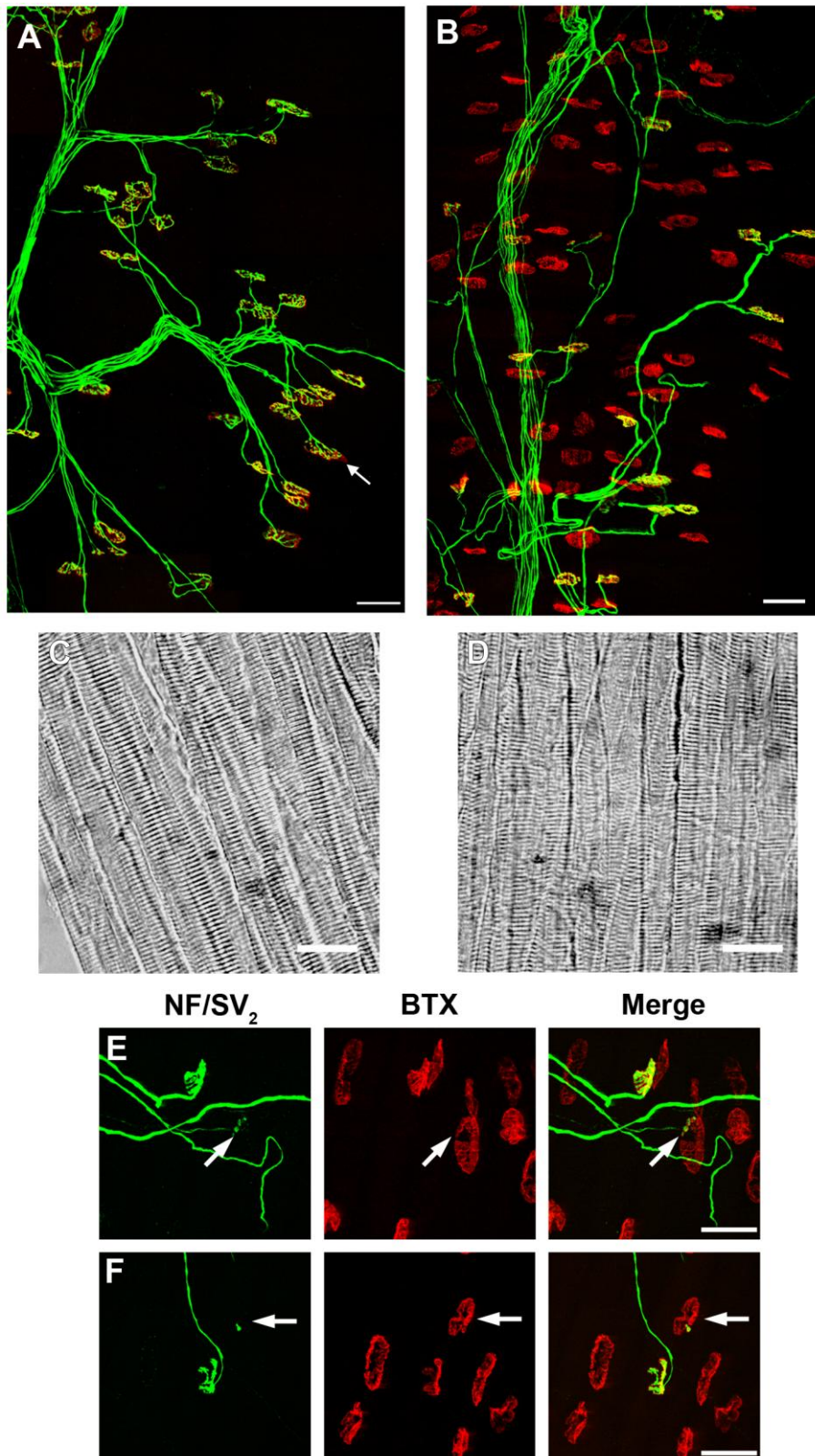


Fig 3.10 2H-2R causes selective α -motor nerve terminal disassembly in lumbrical muscles from *Wld^f* mice aged 5-6wks. Reconstructed confocal projections of parts of *Wld^f* mouse lumbrical muscles aged 5-6wks after being maintained in control conditions (**A**) and following 2H-2R (**B**). In controls (A) most postsynaptic endplates (visualised with BTX: RED) are fully opposed by presynaptic NF/SV₂ immunoreactivity (GREEN) however an endplate (arrow) can be observed to have no opposing NF/SV₂ immunoreactivity in part of its arborisation, i.e., the endplate is partially occupied. This is most likely the result of naturally occurring path-physiological processes. Following 2H-2R, postsynaptic endplates appear unchanged but there is widespread loss of presynaptic NF/SV₂ immunoreactivity. 2H-2R does not appear to affect muscle fibres as phase-contrast micrographs show uniform muscle fibres with clear striations in both control (**C**) and 2H-2R treated *Wld^f* lumbrical muscles (**D**). Confocal projections of a small population of endplates following 2H-2R appear partially occupied by presynaptic NF/SV₂ immunoreactivity (**E&F**). In (**E**) there is asynchronous loss of NF/SV₂ at one endplate (arrow) which is indicative of a withdrawal-like mechanism. In contrast (**F**) shows an endplate (arrow) with fragmented NF/SV₂ immunoreactivity that is more consistent with a Wallerian-like mechanism. Scale bars: 50 μ m

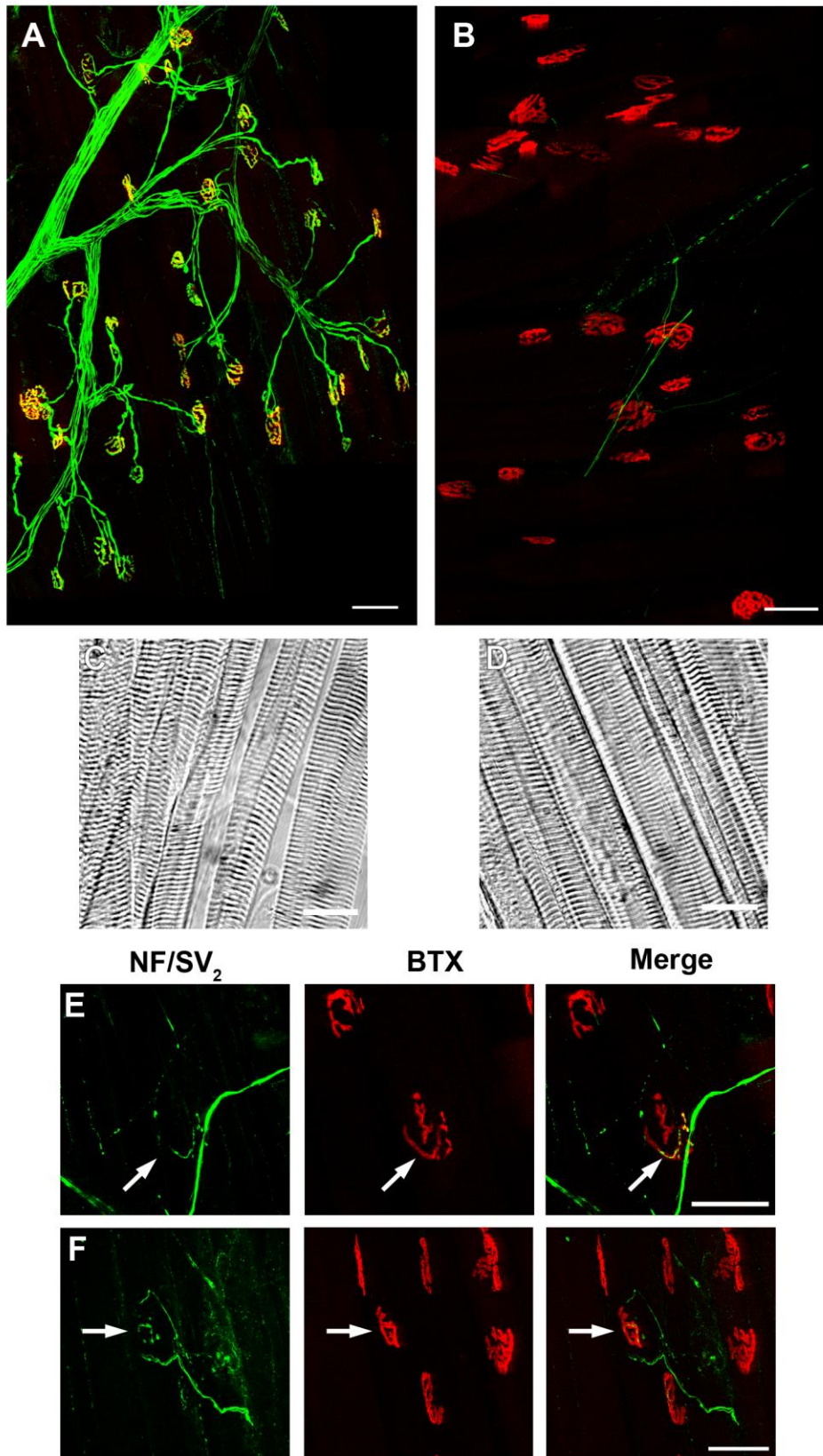


Fig 3.11 2H-2R causes selective α -motor nerve terminal disassembly in TA muscles from *Wld^f* mice aged 5-6wks. Reconstructed confocal projections of parts of *Wld^f* mouse TA muscles aged 5-6wks after being maintained in control conditions (A) or following 2H-2R (B). While postsynaptic endplates (visualised with BTX: RED) appear unchanged, there is widespread loss of presynaptic NF/SV₂ immunoreactivity (GREEN) following 2H-2R. 2H-2R does not appear to affect muscle fibres as phase-contrast micrographs show uniform muscle fibres with clear striations in both control (C) and 2H-2R treated *Wld^f* TA muscles (D). Confocal projections of a small population of endplates following 2H-2R appear partially occupied by presynaptic NF/SV₂ immunoreactivity (E&F). In (E) there is asynchronous loss of NF/SV₂ at one endplate (arrow) which is indicative of a withdrawal-like mechanism. In contrast (F) shows an endplate (arrow) with fragmented NF/SV₂ immunoreactivity that is more consistent with a Wallerian-like mechanism. Scale bars: 50 μ m

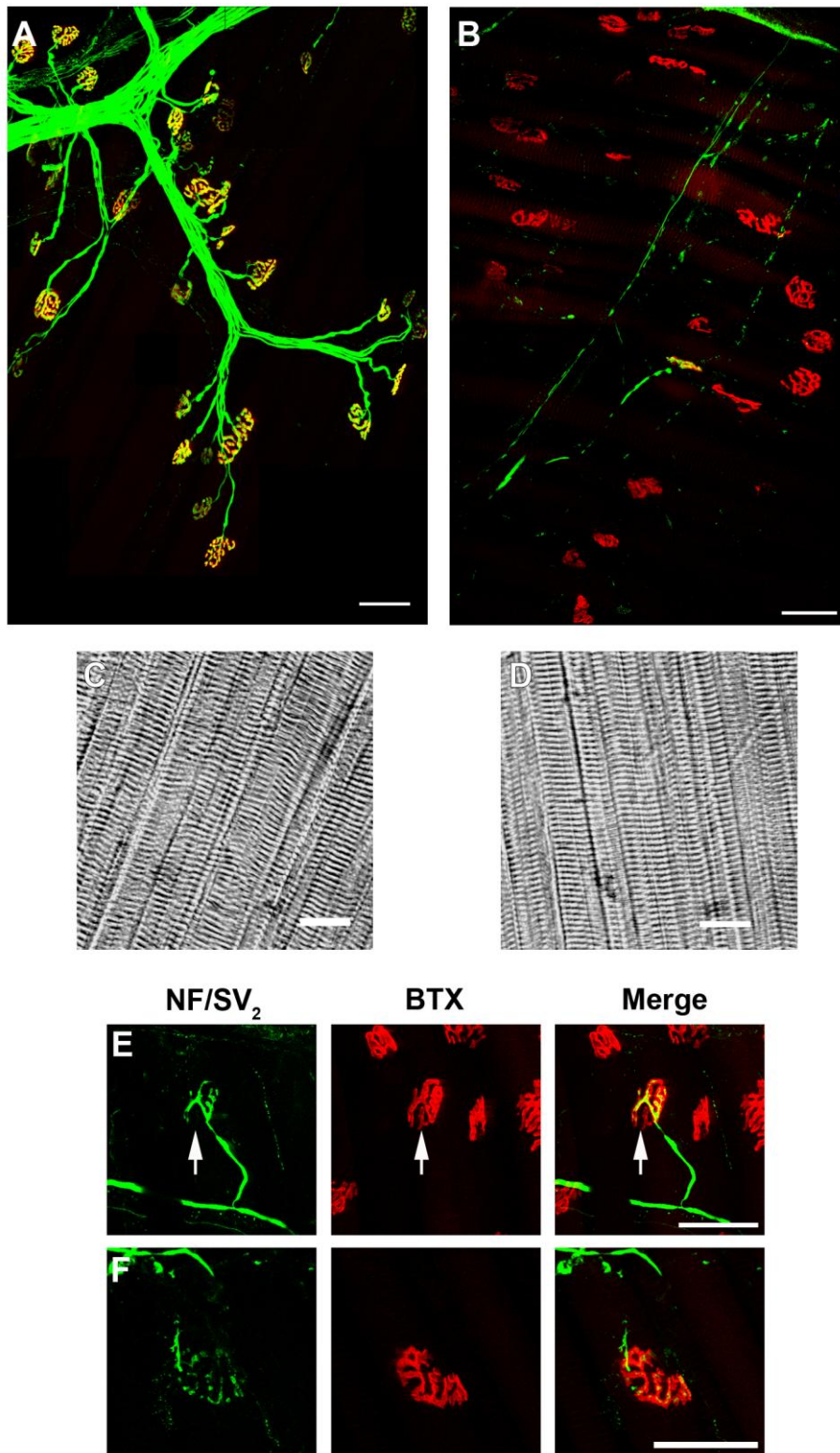


Fig 3.12 2H-2R causes selective α -motor nerve terminal disassembly in TS muscles from *Wld^s* mice aged 5-6wks. Reconstructed confocal projections of parts of *Wld^s* mouse TS muscles aged 5-6wks after being maintained in control conditions (**A**) or following 2H-2R (**B**). While postsynaptic endplates (visualised with BTX: RED) appear unchanged, there is widespread loss of presynaptic NF/SV₂ immunoreactivity (GREEN) following 2H-2R. 2H-2R does not appear to affect muscle fibres as phase-contrast micrographs show uniform muscle fibres with clear striations in both control (**C**) and 2H-2R treated *Wld^s* TS muscles (**D**). Confocal projections of a small population of endplates following 2H-2R appear partially occupied by presynaptic NF/SV₂ immunoreactivity (**E&F**). In (**E**) there is asynchronous loss of NF/SV₂ at one endplate (arrow) which is indicative of a withdrawal-like mechanism. In contrast (**F**) shows an endplate (arrow) with fragmented NF/SV₂ immunoreactivity that is more consistent with a Wallerian-like mechanism. Scale bars: 50 μ m

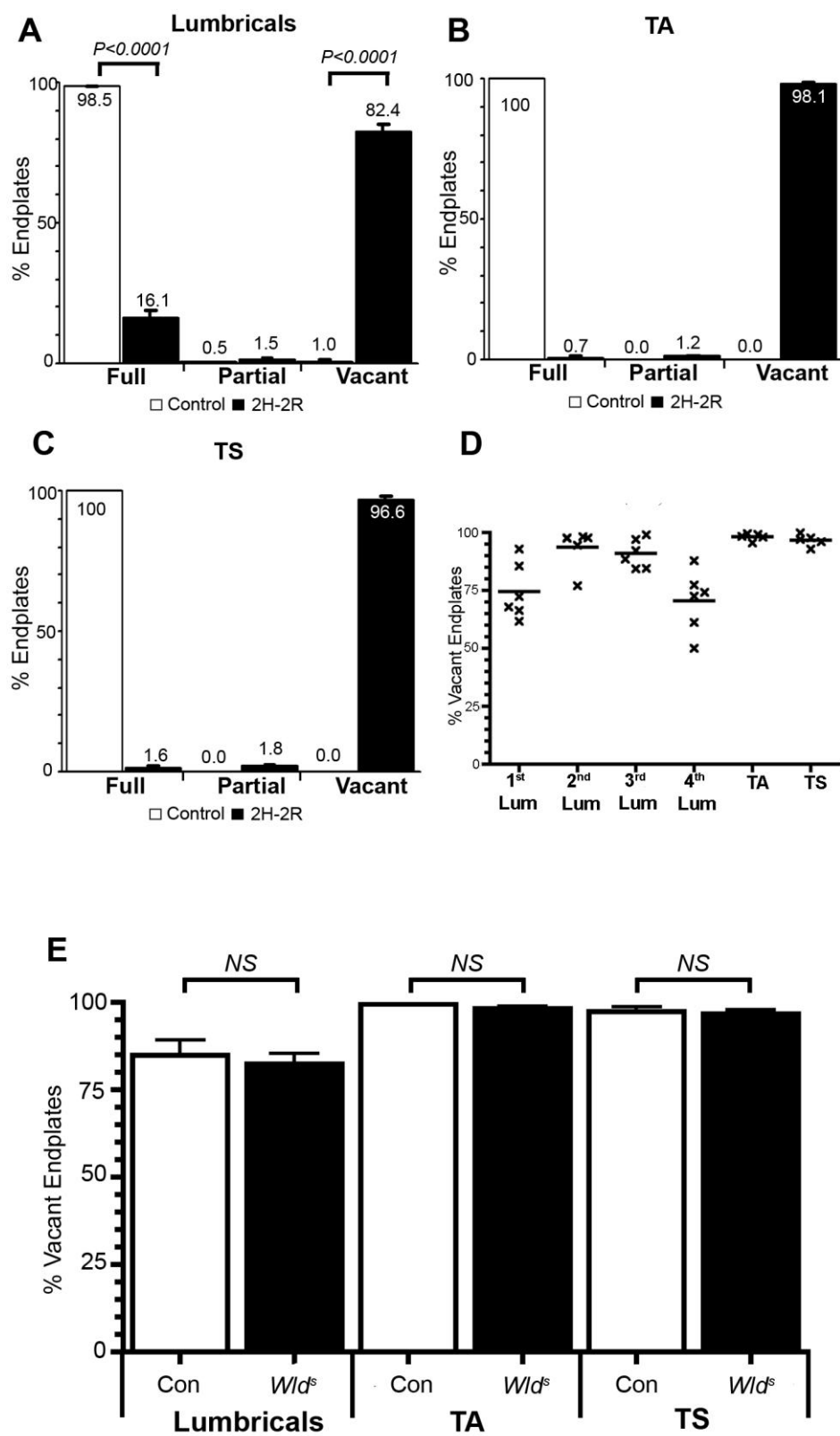


Fig 3.13 Quantification of α -motor nerve terminal response to hypoxia-reperfusion injury shows that the *Wld^f* mutation does not convey any neuroprotection. Bar charts (%mean \pm SE) showing the categorical quantification of α -motor nerve terminal status (i.e., fully-occupied, partially-occupied or vacant) that oppose postsynaptic endplates in lumbrical (**A**), TA (**B**), and TS (**C**) muscles from *Wld^f* mice aged 5-6wks, where 2H-2R treated muscles (black bars) can be compared to controls (white bars). In (**D**), a scatter plot (%mean indicated by line) showing inter-muscle differences in the number of endplates identified with no NF/SV₂ immunoreactivity, i.e., vacant, following 2H-2R injury). Similar to the results described in 8-12wk old mice, and 5-6wk old mice, there were some intra-lumbrical differences in 5-6wk old *Wld^f* mice, but this was not statistically significant. There were also no statically significant inter-muscle differences in 5-6wk old *Wld^f* mice. In (**E**) a bar chart compares the percentage of vacant endplates from all three muscles following 2H-2R in animals aged 5-6wks carrying the *Wld^f* mutation (black bars) and age matched wild type animals (white bars: i.e., data from 5-6wk old animals described above). Statistical analysis shows there was no significant difference between the percentage of vacant endplates following hypoxia-reperfusion injury in wild-type animals and those carrying the *Wld^f* mutation in all three muscles.

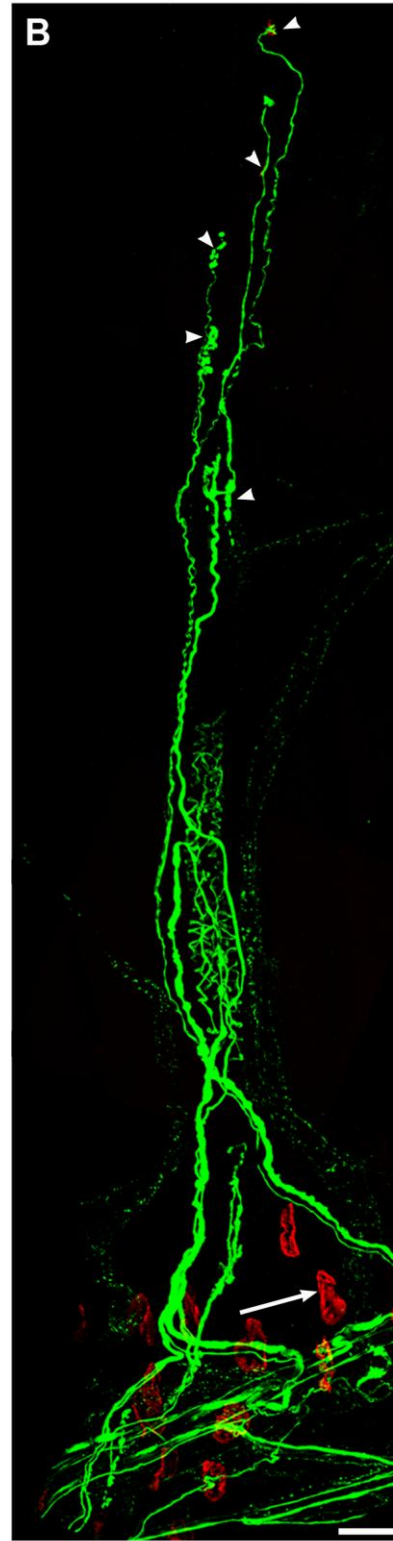
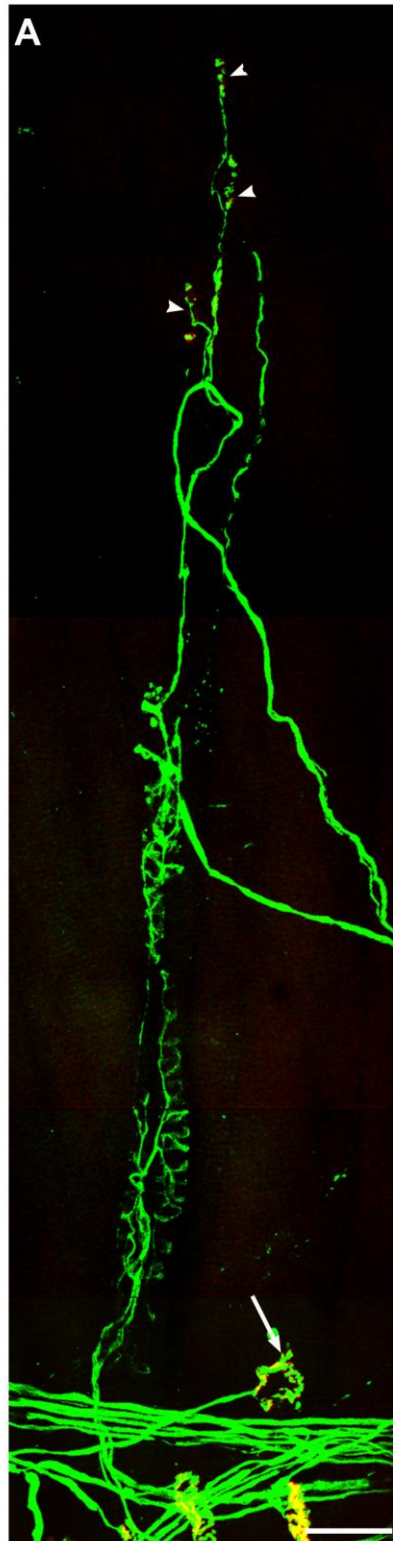


Fig 3.14 the morphology of nerve terminals of the muscle spindle does not appear to be affected by 2H-2R in 8-12wk old mice. Reconstructed confocal projections of complete muscle spindles from a lumbrical muscle after being maintained in control conditions (A) and following 2H-2R (B). In both control and in 2H-2R treated lumbricals, sensory IA/II afferent nerve terminals can be seen to form evenly spaced and regular spiral or half-ring annulospiral endings, indentified in the low-half of (A) and (B) by presynaptic NF/SV₂ immunoreactivity (GREEN). γ - motor nerve terminals form trails of varicosities extending away from the IA/II endings (upper-half of (A) and (B)) and are opposed by small postsynaptic plaques that are visualised with variable intensities of BTX staining (RED: arrowheads). This directly compares with α -motor nerve terminals (arrow) from controls (A) that can be seen to form relatively large and complex arborisations and are opposed by clear postsynaptic staining of the postsynaptic endplate. While there is no obvious sign of pathology in IA/II afferent or γ -motor nerve terminals following 2H-2R (B), there are clear examples of vacant morphologies of α -motor nerve terminals (e.g., arrow). Scale bars: 50 μ m

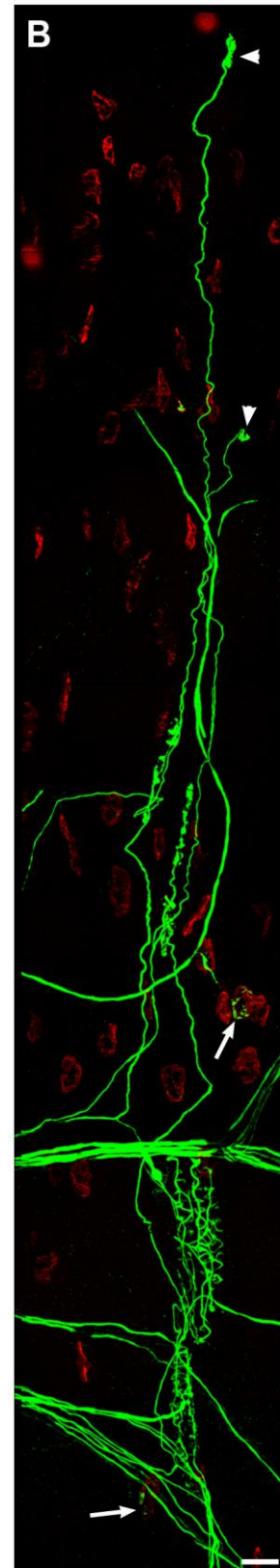
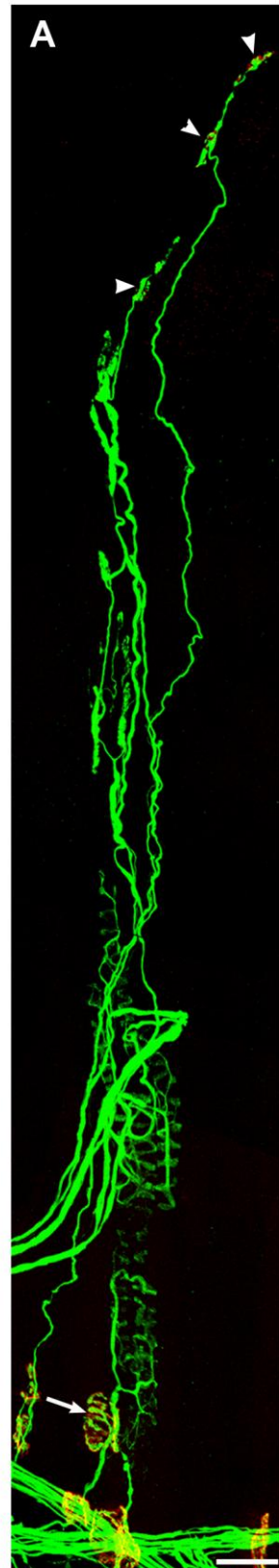


Fig 3.15 The morphology of nerve terminals of the muscle spindle does not appear to be affected by 2H-2R in 5-6wk old mice. Reconstructed confocal projections of complete muscle spindles from a lumbrical muscle after being maintained in control conditions (**A**) and following 2H-2R (**B**). In both control and in 2H-2R treated lumbricals, sensory IA/II afferent and γ - motor nerve terminals can be clearly identified by presynaptic NF/SV₂ immunoreactivity (GREEN) with no apparent signs of pathology. The postsynaptic plaques of the γ - motor nerve terminals (arrowheads) and α -motor nerve terminals are visualised with BTX (RED:). In direct contrast to sensory IA/II afferent and γ - motor nerve terminals, following 2H-2R (**B**) there are clear examples of vacant and partial (arrow) morphologies of α -motor nerve terminals. Scale bars: 50 μ m

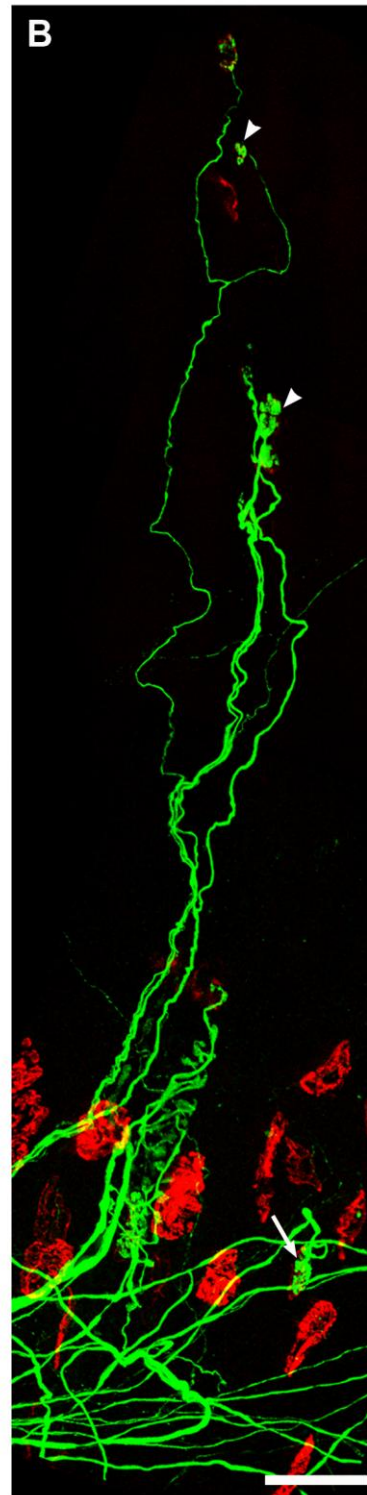
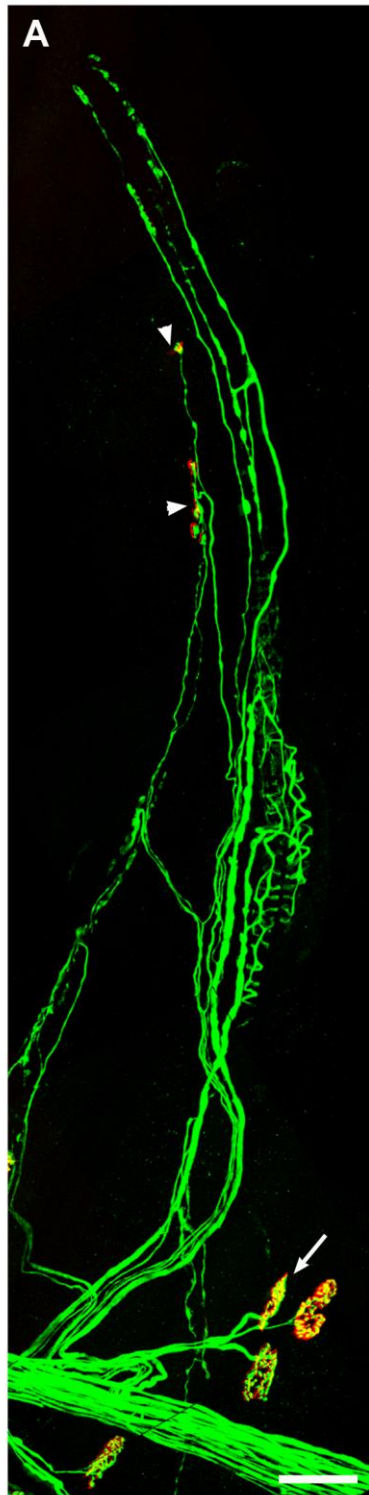


Fig 3.16 The morphology of nerve terminals of the muscle spindle does not appear to be affected by 2H-2R in 5-6wk old *Wld^s* mice. Reconstructed confocal projections of complete muscle spindles from a *Wld^s* lumbrical muscle after being maintained in control conditions (**A**) and following 2H-2R (**B**). The postsynaptic plaques of the γ -motor nerve terminals (arrowheads) and α -motor nerve terminals are visualised with BTX (RED). In contrast to formation of spiral or half-ring annulospiral endings of sensory IA/II afferent that can be observed in (A) by presynaptic NF/SV₂ immunoreactivity (GREEN), in (B) the IA/II afferent nerve terminal appears composed of closely scattered varicosities that is known as a 'flower spray'. Despite this less common form of IA/II afferent nerve terminals, there is again no obvious signs of pathology to either IA/II afferent or γ -motor nerve terminals following 2H-2R. In contrast, following 2H-2R (B) there are clear examples of vacant morphologies of α -motor nerve terminals, with only one endplate (arrow) fully opposed by NF/SV₂ immunoreactivity. Scale bars: 50 μ m

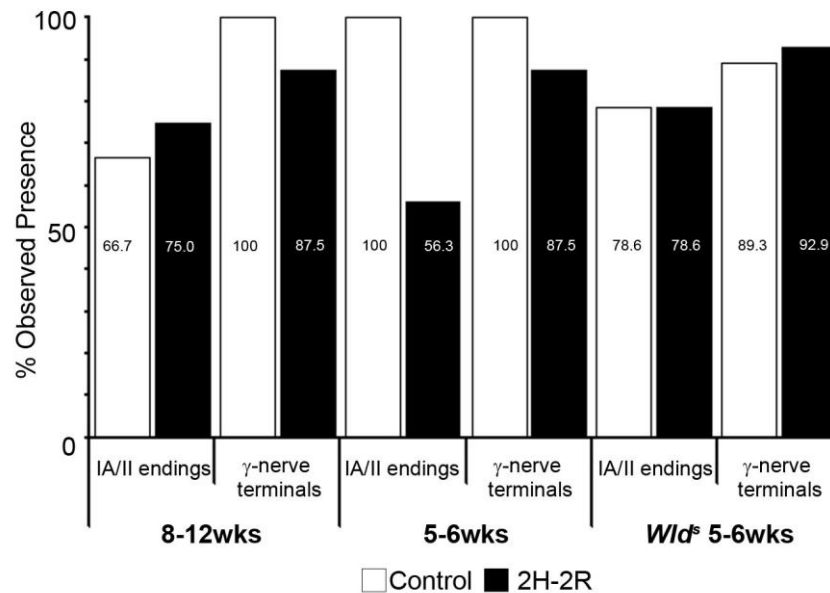


Fig 3.17 Quantification of the presence of IA/II afferent and γ - motor nerve terminals following hypoxia-reperfusion injury Bar chart showing the percentage of lumbrical muscles from 8-12wk, 5-6wk old mice and 5-6wk *Wld^Δ* mice where IA/II afferents and/or γ -motor nerve terminals could be identified without any signs of pathology after being maintained in control condition (white bars) and following 2H-2R (black bars). Even in control conditions, IA/II afferents and/or γ -motor nerve terminals could not always be observed which is most likely due to the poor penetration of antibodies. Without good control data, the observed presence of IA/II afferents and/or γ -motor nerve terminals following 2H-2R is difficult to assess. For example, in 5-6wk old animals the presence of IA/II afferents nerve terminals is seen to decrease following 2H-2R compared to age-matched controls however, in 8-12wk old animals the reverse occurs. This data on its own therefore provides little information on the vulnerability of IA/II afferents or γ -motor nerve terminals to hypoxia-reperfusion injury, or the ability of *Wld^Δ* mutation to confer protection.

3.4 Discussion

Using the novel hypoxia-reperfusion model described in Chapter 2, I present further evidence in support of the finding that α -motor nerve terminals are highly and selectively vulnerable to hypoxia-reperfusion injuries. In this Chapter I have presented morphological evidence that shows α -motor nerve terminals from TA and TS muscles undergo disassembly following 2hr hypoxia-2hr reperfusion and that functional disruption of synaptic vesicle release/recycling probably precedes loss of morphology. I have identified important age dependent, inter-muscular differences affecting the severity of α -motor nerve terminal pathology between lumbrical, TA and TS muscles. I have also provided some preliminary evidence suggesting that hypoxia-reperfusion is highly selective for α -motor nerve terminals with γ -motor nerve terminals and IA/II sensory endings, which innervate the intrafusal muscle fibres of the muscle spindle, being more resistant. Finally, I have shown that the rapid loss of α -motor nerve terminals following hypoxia-reperfusion injury occurs via mechanisms distinct from classic Wallerian degeneration pathways, as the *Wld^Δ* mutation failed to protect α -motor nerve terminals.

3.4.1 Hypoxia-reperfusion causes functional impairment followed by selective morphological signs of on-going α -motor nerve terminal disassembly.

Following on from the results presented in Chapter 2 where I showed that 82.5% of α -motor nerve terminals from lumbrical muscles had lost their NF/SV₂ immunoreactivity following 2H-2R, I have now also shown significant loss of NF/SV₂ immunoreactivity from 57.9% and 27.7% of α -motor nerve terminals and distal axons from TA and TS muscles respectively following 2H-2R. Consistent with results obtained from lumbrical muscles, I observed a small population of terminals, 4.8% and 3.2% in TA and TS muscles respectively, showing evidence of on-going disassembly via both Wallerian-like degeneration and withdrawal-like processes. In addition to this morphological evidence, I have shown additional data that indicates that loss of morphology in α -motor nerve terminals from TA and TS muscles is preceded by functional loss of synaptic vesicle release/recycling. This is indicated by the 37% and 69% of α -motor nerve terminal that still retain full NF/SV₂ immunoreactivity in TA and TS muscles respectively but complete failure of the vital dye FM1-43FX to label α -motor nerve terminals from either muscle following 2H-2R. Functional changes to presynaptic α -motor neurons following hypoxia have previously been shown with a massive increase in spontaneous vesicle release (MEPP frequency: Hubbard and Loynning 1966; Anwyl and Ling 1983; Nishimura 1986; Bukharaeva et al. 2005) followed by progressive decreases in evoked vesicle release (EPP) leading to complete failure to elicit skeletal muscles contraction by stimulating nerve stumps (Krnjevic and Miledi 1959; Hubbard and Loynning 1966; Bazzy 1994; Zhu et al. 2006). Functional loss is known to occur in CNS ischaemic injuries both *in vivo* and *in vitro* and appears to

coincide with conduction block, a reduction in the number of synaptic vesicles and followed by loss of key synaptic proteins critical for synaptic vesicle mobilisation, release and recycling that later leads to whole cell death (von Lubitz and Diemer 1983; Lipton 1999; Nishimura et al. 2000; White et al. 2000; Ishimaru et al. 2001; Bolay et al. 2002; Jourdain et al. 2002; Jung et al. 2004; Kovalenko et al. 2006). Similar temporal changes in function and morphology (where functional changes precede changes to gross morphology) are also known to occur in other neurodegenerative disease processes including axotomy induced Wallerian degeneration and in several peripheral neuropathies (e.g., Miledi and Slater 1968; 1970; Miura et al. 1993; Frey et al. 2000; LoPachin et al. 2002; Ferri et al. 2003; Gillingwater and Ribchester 2003; LoPachin et al. 2003; Pun et al. 2006; Wishart et al. 2006; Murray et al. 2008a; Murray et al. 2008b). It is possible that the loss of synaptic function described here, is related in part to the loss of SV₂ protein immunoreactivity. The role of SV₂ is still unknown but it has a structure characteristic of a trans-membrane transporter, possibly transporting Ca²⁺ ions, and appears to be involved in priming docked vesicles for release (Xu and Bajjalieh 2001; Sudhof 2004). Interestingly, two ATP binding sites have just been identified on the SV₂ protein that appear to modify SV₂ activity, suggesting a direct link between cellular energy levels and vesicle release (Yao and Bajjalieh 2008). From the data presented here however, functional loss did not completely coincide with loss of synaptic vesicle proteins (SV₂) which suggests that either SV₂ function is lost prior to its loss of immunoreactivity and/or loss of function is due to the loss of other critical mediators of synaptic vesicle release/recycling. This will need to be the subject of further study. This data is however consistent with the general conclusion from Chapter 2 that hypoxia-reperfusion triggers selective α -motor nerve terminal disassembly and further indicates that there is a complex set of both functional and structural spatiotemporal events leading to α -motor nerve terminal pathology. Further experiments are required to accurately define these spatiotemporal characteristics, particularly as assessment of α -motor nerve terminal function with the vital dye FM1-43 is known to be a relatively insensitive means of determining functional impairment (Rogozhin et al. 2008). It would be of key importance to determine when functional impairment begins and if, and when, this is followed by complete functional loss and how this correlates with structural changes. It would also be of interest to investigate if there is an increase in vesicle release prior to functional block as indicated in previous studies (Krnjevic and Miledi 1959; Hubbard and Loyning 1966; Anwyl and Ling 1983; Nishimura 1986; Bazzy 1994; Bukharaeva et al. 2005; Zhu et al. 2006). Further studies combining more detailed structural analysis (both morphological and ultrastructural) with more sensitive functional analysis using electrophysiology would help in pinpointing the exact course of cellular events. Such studies may provide further insight into the cellular/molecular mechanism governing hypoxia-reperfusion injury.

3.4.2 Is α -motor nerve terminal disassembly in response to hypoxia-reperfusion independent of Wallerian Degeneration?

The serendipitous discovery of the spontaneous *Wallerian degeneration slow* (*Wld^s*) mutation in the late 1980's (Lunn *et al.* 1989) has since been shown to delay axotomy induced Wallerian-degeneration of axons and α -motor nerve terminals by at least 3days *in vivo* (Tsao *et al.* 1994; Ribchester *et al.* 1995; Gillingwater *et al.* 2002; Gillingwater *et al.* 2003; Parson *et al.* 2004). The *Wld^s* mutation has also been used to convey neuroprotection and demonstrate the importance of Wallerian degeneration in a range of CNS and PNS neuropathies including; disruption of microtubules (vincristine neuropathy: (Luduena *et al.* 1986; Wang *et al.* 2000; Wang *et al.* 2001), progressive motor neuropathy (Ferri *et al.* 2003; Simonin *et al.* 2007), genetic dysmyelination disorder (Samsam *et al.* 2003), axonal dystrophy (Mi *et al.* 2005), autoimmune encephalomyelitis (a multiple sclerosis model : (Kaneko *et al.* 2006), traumatic brain injury (Gillingwater *et al.* 2006a), glaucoma (Howell *et al.* 2007; Beirowski *et al.* 2008) and Parkinson's diseases (Sajadi *et al.* 2004; Hasbani and O'Malley 2006). Despite observations of Wallerian-like morphology in α -motor nerve terminals following 2H-2R and previous studies identifying Wallerian-like morphology of α -motor nerve terminals following *in vivo* ischaemia (Diwan and Milburn 1986), the *Wld^s* mutation did not provide any overall protection to α -motor nerve terminals in the model system presented here. This would suggest that despite some morphological similarities with Wallerian-degeneration, the mechanism(s) that govern nerve terminal disassembly following hypoxia-reperfusion are independent of pathways that govern Wallerian degeneration. Further to the discussion in Chapter 2 (section 2.4.2) the finding that the *Wld^s* mutation does not convey protection demonstrates that the observed α -motor nerve terminals pathology is very unlikely to involve a contribution from axotomy/mechanical trauma of the distal nerve that occurred during dissection. These results would also suggest that there are important differences between the response of α -motor nerve terminals and neurons in CNS where it has been shown that the *Wld^s* mutation caused up to a ~30% reduction in ischaemic pathology 72hr after brief cerebral ischaemic insult (Gillingwater *et al.* 2004). This difference between the CNS and α -motor neurons may however be due to the slightly different pathological stimuli to which each was subjected. In this study, *ex vivo* α -motor nerve terminals were subjected to a very acute, rapid hypoxic-reperfusion stimulus while Gillingwater *et al.* 2004 showed *Wld^s* mediated protection against chronic, delayed neuronal death 72hr after a 17min ischaemic insult *in vivo*. Ischaemia-reperfusion injuries *in vivo* encompass a whole range of potentially pathological stimuli including hypoxia, hypoglycaemia, acidosis and ionic gradient breakdown, inflammation, excitotoxicity, elevated CO₂, toxic metabolite build-up and ROS (see Chapter 2 and/or section 1.6.1). The model system developed here specifically aimed to reduce as many of these co-stimuli associated with *in vivo* ischaemia-reperfusion injuries as possible to examine only the acute effects of changing O₂ tension. It is possible that the different pathological stimuli between the model system used here and that used by Gillingwater *et al.* (2004), may have altered the effectiveness of the *Wld^s* mutation to confer protection. If α -motor nerve terminal disassembly following hypoxia-reperfusion injury is not governed by

Wallerian-degeneration pathways, then what mechanism(s) do mediate nerve terminal disassembly? As discussed in Chapter 2, observations of on-going α -nerve terminal disassembly (partial morphologies) indicated that both Wallerian degeneration and withdrawal may have been the consequence of spatiotemporal activation of a singular unknown/recognized mechanism (possibly necrotic), which due to the relatively large size of α -motor nerve terminals gave the morphological appearance of multiple mechanisms. It is possible that such a mechanism is therefore not modulated by the *Wld^s* mutation. Again, as discussed in Chapter 2, activation of multiple mechanisms of cell death characterises CNS ischaemia-reperfusion injuries and may also change during the progression of pathology. White *et al.* (2000) suggest that because hypoxia/ischaemia-reperfusion injuries involve so many complex co-stimuli this results in multiple mechanisms of cell death but, because of this, inhibition or manipulation of a singular mechanism to ameliorate hypoxia/ischaemia reperfusion injuries are unlikely to be successful. Rather, once cellular damage reaches a threshold where cell death is inevitable (or in this case nerve terminal disassembly) an inhibited mechanism will be bypassed and cell death will continue down another route. While invariably this should cause a change in morphological phenotype of cell death (Portera-Cailliau *et al.* 1997; Ferrari *et al.* 1999; Fujikawa 2000; Northington *et al.* 2007), without thorough examination of a range of morphological markers the changes in morphology that indicate a change in mechanism may be relatively subtle, particularly if cell death types really do exist on a morphological and mechanistic continuum (Fujikawa 2000; Yakovlev and Faden 2004). In this study, morphological observations of α -motor nerve terminals were based on NF/SV₂ immunoreactivity and while no obvious differences in the extent and range of partial morphologies were noted between wild-type animals and *Wld^s* animals, this protocol may not have picked up other subtle changes in morphology than would indicate the *Wld^s* mutation modulated rather than prevented α -motor nerve terminal response. There is also the distinct possibility that the neuroprotective effect of the *Wld^s* mutation was ineffective against the 2H-2R stimulus. How the *Wld^s* mutation confers axonal and synaptic protection is still unknown but one of the active protein products produced by *Wld^s* mutation is Nmnat1, an important enzyme in the synthesis and regeneration of NAD⁺ (Conforti *et al.* 2000; Mack *et al.* 2001; Raffaelli *et al.* 2002; Adalbert *et al.* 2005; Sasaki *et al.* 2006). NAD⁺ is a vital metabolic substrate that acts as an electron acceptor/donor to fuel aerobic respiration (see Fig 1.1) whose levels can be modulated by Nmnat1 (Raffaelli *et al.* 2002). Although the full *Wld^s* protein product appears to be necessary to convey neuroprotection (Mack *et al.* 2001; Gillingwater *et al.* 2002; Adalbert *et al.* 2005; Conforti *et al.* 2007b) the Nmnat1 portion alone, as well as exogenous application of NAD⁺ can convey some protection to neurons (Mack *et al.* 2001; Ikegami and Koike 2003; Araki *et al.* 2004; Wang *et al.* 2005; Kaneko *et al.* 2006; Sasaki *et al.* 2006; Sasaki *et al.* 2009a; Sasaki *et al.* 2009b; Yahata *et al.* 2009). Further to this, recent proteomic analysis has shown that of the 16 major changes in protein expression identified in *Wld^s* mice, 8 of these proteins are directly linked to or located in the mitochondria (Wishart *et al.* 2007). This would suggest that the *Wld^s* mutation confers its neuroprotective effect, at least in part, via modifications to mitochondria and bioenergetic pathways (Mack *et al.* 2001; Ikegami and Koike 2003; Araki *et al.* 2004; Kaneko *et al.* 2006; Sasaki *et al.* 2006; Wishart *et al.* 2007; Yahata *et al.* 2009). It seems

possible that in response to a pathological insult aimed directly at metabolic pathways and mitochondria, such as hypoxia/ischaemia, that the *Wld^s* neuroprotective mechanism may be ineffective or by-passed. This possibility that *Wld^s* mediated neuroprotection is linked but upstream of direct mitochondrial impairment has been previously suggested (Coleman 2005) based on the failure of the *Wld^s* mutation to prevent degeneration of cultured superior cervical ganglia following uncoupling of the mitochondrial respiratory chain with a cyanide derivative (Ikegami and Koike 2003). It is interesting to note that in many of the studies that describe a failure of the *Wld^s* mutation to convey neuroprotection, the pathological stimuli have direct links to respiratory pathways and mitochondria. This includes the familiar model of ALS caused by mSOD1, a powerful ROS scavenging enzyme (Vande Velde *et al.* 2004; Fischer *et al.* 2005) and in hereditary spastic paraplegia (Edgar 2004) where the disease has been linked to the mitochondrial protein, paraplegin, and impaired oxidative phosphorylation (Beal 2000; Ferreira *et al.* 2004). The *Wld^s* mutation has also failed to convey neuroprotection in response to other stimuli not linked to respiratory pathways and mitochondria: spinal muscular atrophy (Kariya *et al.* 2009) caused by lowered protein products of the *survival motor neuron* gene which have been suggested to alter mRNA transport and gene splicing (Burghes and Beattie 2009) and following application of botulinum C neurotoxin (Berliocchi *et al.* 2005). Overall, the failure of the *Wld^s* mutation to protect α -motor nerve terminals from hypoxia-reperfusion may not necessarily mean that Wallerian degeneration pathways are not activated. This study demonstrates the difficulty in attempting to determine the mechanisms of nerve terminal disassembly by morphological analysis alone, particularly as different morphological mechanisms have yet to be linked to a clear underlying biochemical mechanism, a problem that has also been highlighted in CNS ischaemic injuries (Lipton 1999; Fujikawa 2000; Hand and Menze 2008). Further studies investigating the mechanism(s) responsible for disassembling motor nerve terminals following hypoxia-reperfusion injury, as well as nerve terminals loss in response to other pathological stimuli are therefore required. Further investigation are also needs to address how the *Wld^s* mutation confers its neuroprotective effect.

3.4.3 Age and muscle type/location difference in the α -motor nerve terminal response to hypoxia-reperfusion injury

When the affect of 2H-2R on predominantly slow-fibre type muscles (TA and TS: e.g., Iscoe 1998; Hodges 1999; Murray *et al.* 2008a) was compared with that of the predominantly fast-fibre type muscles (lumbricals: e.g., Betz *et al.* 1990; Murray *et al.* 2008a), I identified significant differences in the extent of pathology with the α -motor nerve terminals from lumbrical muscles being more vulnerable than those from TA or TS muscles. This indicates that different populations of α -motor nerve terminals are selectively vulnerable/resistant to hypoxia- reperfusion injury. Differences in the selective vulnerability/resistance between and within populations of neurons is a key feature of CNS ischaemia-reperfusion injuries where, for example, neurons in region CA1 in the hippocampus are

more vulnerable than CA2 and 3 in rats and gerbils (e.g., Nakano *et al.* 1990; Ordy *et al.* 1993; Lipton 1999; Gillingwater *et al.* 2004). The mechanisms that govern this selective vulnerability in CNS hypoxia/ischaemia-reperfusion injuries are poorly understood, but are probably due to the large number of complex variables that can influence hypoxia/ischaemia tolerance. For example, Lipton (1991) identified at least 21 different factors that have been shown to be, at least in part, responsible for the selective vulnerability of CA1 hippocampal neurons. The results presented here indicate that the selective vulnerability/resistance of α -motor nerve terminals in response to hypoxia-reperfusion injury is influenced by factors relating to muscle-fibre subtype. When the metabolic properties of muscle fibres are considered however, the increased vulnerability of α -motor nerve terminals innervating predominantly fast-twitch muscles is intriguing. Fast-twitch fibres rely on predominantly anaerobic, oxygen independent pathways for the supply of ATP for rapid but short-term contraction, while slow-twitch muscles predominantly rely on oxygen dependent, aerobic pathways for slow but tonic contraction (Ranvier 1874; Ogata and Mori 1964; Ogata and Yamasaki 1985; Rolfe and Brown 1997; Kernell 2006). These different patterns of activity between muscle fibres will also be shared by their innervating α -motor neurons and others have already highlighted that there is a reciprocal relationship between the anatomical and functional parameters of α -motor neurons and skeletal muscle fibre subtypes (Wood and Slater 1997; Bewick *et al.* 2004; Kernell 2006; see section 1.4.2). Very little work has been carried out to address the possibility that α -motor neurons and skeletal muscle fibres also share a reciprocal metabolic relationship but a decreased reliance on aerobic pathways is suggested by the lower levels of SDH in α -motor neurons innervating fast-twitch muscles (Campa and Engel 1971; Donselaar *et al.* 1986; Ishihara *et al.* 1988; Chalmers and Edgerton 1989; Chalmers *et al.* 1992; Ishihara *et al.* 1995; Nakano *et al.* 1997). Overall this would suggest that fast-twitch muscles and therefore, possibly their α -motor neurons have increased anaerobic provisions for sustaining ATP supply and be more resistant to hypoxic/ischaemic insults. This has been found to be the case in some studies concerning the vulnerability of skeletal muscle fibres to ischaemia-reperfusion injuries *in vivo* where fast-fibre type skeletal muscles are more resistant (Chervu *et al.* 1989; Mars and Gregory 1991; Pedowitz *et al.* 1992; Awerbuck *et al.* 1994; Chan *et al.* 2004) but, conversely others have found that slow-twitch muscles are more resistant (Idstrom *et al.* 1990; Woitaske and McCarter 1998). The results presented here from 8-12wk old animals and in previous studies following application of tourniquets in rats (Tombol *et al.* 2002) and mice (David *et al.* 2007) indicate that α -motor nerve terminals from predominantly fast-twitch muscles are the most vulnerable. This would overall suggest that despite the increased provisions for O₂ independent supply of ATP in fast-twitch muscles and the possibly that this also applies to their innervating α -motor neurons, there are other factors determining their vulnerability. The selective vulnerability of α -motor nerve terminals from fast-twitch muscles is consistent with findings from amyotrophic lateral sclerosis (Kawamura *et al.* ; Frey *et al.* 2000; Pun *et al.* 2006), poliomyelitis (Hodes 1949; Hodes *et al.* 1949) and during the normal ageing process (Hashizume *et al.* 1988). The reasons underling this selective vulnerability have been suggested to be the consequence of the larger size and the formation of larger

motor units by α -motor neurons that innervate fast-twitch muscles (Campa and Engel 1971; Kernell 2006) which overall increases stress and lowers tolerance to pathological stimuli (Kernell 2006). In spinal muscular atrophy however, α -motor nerve terminals innervating slow-twitch muscles have been shown to be more vulnerable (Murray *et al.* 2008a). This would indicate that rather than differences based purely on muscle fibre type, selective vulnerability/resistance may also vary depending on the pathological stimulus (Murray *et al.* 2008a). Further to this, other studies have also identified differences in motor neuron properties and pathological responses between and within affected muscles that show correlation with speed at which developmental synaptogenesis/maturation took place, the ability of terminal Schwann cells to express the chemorepellent Semaphorin 3A, the capacity of α -motor nerve terminals to sprout, as well as anatomical positioning/topographic mapping of innervating α -motor neurons (Duchen 1970; Laskowski and Sanes 1987; Miura *et al.* 1993; Frey *et al.* 2000; Pun *et al.* 2002; Santos and Caroni 2003; Schaefer *et al.* 2005; De Winter *et al.* 2006; Burns *et al.* 2007a; Murray *et al.* 2008a). A further possibility is that the muscle type differences described here in response to hypoxia-reperfusion injury may relate to their anatomical position within the body and length of the motor neurons that innervate each of these muscles. It is thought that mitochondria, the principal target of hypoxic injuries, undergo ageing and accumulation of oxidative damage as they are transported to nerve terminals. This process reduces their Ca^{2+} buffering capacity and increases the risk of mitochondrial rupture that leads to increases in cell death stimuli and may be, in part, responsible for the general vulnerability of nerve terminals in a range of pathological conditions (Davey *et al.* 1998; Brown *et al.* 2006; Naga *et al.* 2007). This would imply a hierarchy of pathological vulnerability, with nerve terminals of the longest neurons being more vulnerable (Davey *et al.* 1998) to hypoxic/ischaemic insults than those of shorter neurons. This is seen in the results represented here, where at 8-12wks of age, the lumbrical muscles with the longest α -motor neurons were observed to be the most vulnerable to 2H-2R, while TS muscles that had the shortest α -motor neurons were the least vulnerable to 2H-2R. TA muscles occupied the middle ground in terms of overall motor neuron length and extent of α -motor nerve terminal pathology. This would help explain the observed differences between the extent of α -motor nerve pathology in TA and TS muscle (58.9% and 27.7% loss of NF/SV₂ in TA and TS muscles respectively) as they are both composed of predominantly slow-twitch muscle fibres. It must be pointed out however that the difference between the mean number of vacant endplates from slow-twitch TA muscles was found not to statistically differ from either that of the lumbrical muscles or the TS muscles. This is probably due to a combination of factors including the lower *n* number (of both TA and TS muscles compared to lumbricals) but also the low power and conservative nature of the statistical testing that would have increased the chance of producing a false-negative *P* value (Dytham 2003). In combination with the high degree of variability in the observed responses of both TA and TS muscles, overall makes the true nature of the differences between TA and TS muscle, and the difference between those muscles and the lumbrical muscles difficult to accurately determine. The more variable response of the TA and TS muscles, as well as the observed variability in the 4th deep lumbrical noted here and in Chapter 2,

may however further suggest that there are additional (and perhaps more important) factors like those already discussed above governing the vulnerability of α -motor nerve terminals to hypoxic/ischaemic injury than fibre type alone. These results may also be a reflection of the over simplified classification of muscle fibre subtype used in this study (i.e., either fast- or slow-twitch) as there are numerous intermediate fibre subtypes, depending on classification method, between these two fast- and slow-twitch extremes (Close 1972; Kernell 2006). While both TA and TS are composed of predominantly slow-muscle fibres (Iscoe 1998; Hodges 1999; Murray *et al.* 2008a), it is possible that there are subtle differences in the mix of muscle fibre sub-types between these muscles that perhaps, in part, caused the observed differences in response. There may be also factors that are more related to other, more discreet functional parameters: TA is a postural muscle while TS is a respiratory muscle that will each have unique patterns of activity that perhaps results in some distinctive properties between these muscles, with further differences observed between them and the fast-twitch lumbrical muscles used for flexing the digits (Wood and Slater 1997; Iscoe 1998; Polla *et al.* 2004) that may affect relative vulnerabilities. Overall, the factors that influence α -motor nerve terminal vulnerability/resistance to pathological stimuli are still almost entirely unknown and it may be that generalizations on the extent of vulnerability based on a single variable, such as fibre type, are an oversimplification. As well as relative vulnerabilities based on the type of pathological stimulus, rates of development and fibre type (Murray *et al.* 2008a), other variables including, anatomical location, functional attributes, genetic differences, metabolic properties, level of maturity as well as environmental factors may all contribute to produce unique and dynamic subpopulations of motor neurons between and within different skeletal muscles that differ greatly in their responses to different pathological stimuli. Identification of some of these variables that contribute to selective vulnerability/resistances is important not only to identify the most vulnerable subpopulations of neurons in neuropathological conditions but also to identify novel therapeutic strategies than can improve their resistance. The hypoxia-reperfusion model system developed here provides an opportunity to study some of these variables. Further studies using a greater range of muscle where α -motor nerve terminals pathology can be directly correlated with fibre-type should be used to confirm/disprove the finding of fibre-type differences that are indicated here. The model system presented provides an ideal opportunity to investigate other variables as the results shown here indicate a distinct age-dependent component to the fibre-types difference. The surprising and unexplainable finding that in juvenile mice aged 5-6wks there was no apparent muscle type differences suggests that there are rapid and fundamental changes in α -motor nerve terminals between 5-6wks and 8-12wks of age that led to the observed increased resistance to hypoxia-reperfusion injury in TA and TS muscle in adult mice. Such dramatic differences could perhaps be expected if a comparison had been carried out between adult mice and those in the first few post-natal weeks for example, but at 5-6wks of age these animals would have already adopted most, in not all, of their adult behavioural patterns including sexual maturity. It has also been noted that children and young adults tend to be more resistant to tourniquet induced ischaemia-reperfusion injuries (Bruner 1951), contrasting to the results presented here where the TA and TS muscles in juvenile mice were

more vulnerable. If the data from 8-12wk old animals reflects adult patterns of α -motor nerve terminal vulnerability, it is curious to note that the lumbrical muscles appear to adopt their mature pattern of vulnerability before 5-6wks while the respiratory/postural TS/TA muscles do not until 8-12wks. The very narrow time frame of these observed changes provides an ideal starting point to investigate the factor or factors acting synergistically that influence the vulnerability/resistance to hypoxia-reperfusion injury. Further studies that examine the extent of pathology with age (e.g., early postnatal versus >12mth) may also highlight other significant age-related differences and their critical time points following hypoxia-reperfusion injuries. Such studies may later also provide insights into the factors that govern selective vulnerability seen in response to hypoxia-reperfusion injury and also other pathological stimuli.

3.4.4 Preliminary evidence suggests that muscle spindles may be more resistant to hypoxia-reperfusion injury

In support of the general finding of selective vulnerability of α -motor nerve terminals, I also report preliminary evidence that may indicate γ -motor nerve terminals and IA/II sensory endings innervating intrafusal muscle fibres (collectively, the muscle spindle) of the lumbrical muscles may be more resistant to hypoxia-reperfusion injury. This is based on the preliminary finding that the observed frequency of IA/II sensory endings and γ -neuromuscular junctions that still appeared with normal morphology (determined by NF/SV₂ immunoreactivity) following 2H-2R was >56% compared to <16% of α -motor nerve terminals from lumbrical muscles. Furthermore, on no occasion (either in controls or following 2H-2R) were IA/II sensory endings and γ -neuromuscular junctions observed to have any obvious signs of pathology. Due to difficulty in interpreting the reliability of the negative data from study (where IA/II sensory endings and γ -neuromuscular junctions could not be observed, most probably due to immunohistochemical protocol staining caveats) this does however remain a preliminary finding and highlights the need for this to be the subject of further study. Differences between the relative vulnerabilities/resistance of α -, γ -, and IA/II nerve terminals have however been reported following axotomy (Sherrington 1893; Tower 1932; Boyd 1962; Barker and Ip 1966; Barker *et al.* 1970; Taylor *et al.* 2005), latrotoxin (Queiroz and Duchon 1982), normal ageing (Swash and Fox 1972) in the gracile axonal dystrophy mutant mouse (Takagi *et al.* 1996) and in their responses to local anaesthetics, low Na⁺ concentrations and hypothermia (Matthews 1964). Some authors have reported loss of sensory action potentials after vascular occlusion in the hind limbs of the rat (Kawamura *et al.* 2006) and loss of proprioception, paraesthesia ('pins and needles') touch and vibration sensation have been noted in human surgical patients where tourniquets have been used (Eckhoff 1931; Moldaver 1954). The few studies that have examined the pathological response of muscle spindles to hypoxia/ischaemia-reperfusion injuries do indicate that the nerve terminals of the muscle spindle are vulnerable and that there are differences in the selective vulnerabilities/resistance of α -, γ -, and IA/II nerve terminals. Ischaemia induced by aortic compression in the rabbit did not

affect IA/II sensory endings but triggered γ -nerve terminals to degenerate (Cipollone 1898 as described by Matthews 1964). In a more recent study, γ -motor nerve terminals withdrew from their postsynaptic endplates following transection of the vascular supply to rat soleus muscles, while IA/II sensory endings and α -motor nerve terminals underwent Wallerian-like degeneration. However, temporal analysis between these differences response was not documented (Diwan and Milburn 1986). In this study, while I could not show any conclusive pathological response in IA/II sensory and γ -motor nerve terminals, the preliminary evidence may indicate that the sensory and motor nerve terminals of the muscle spindle may be more resistant than those of α -motor nerve terminals following 2H-2R. The reasons underlying this possible selective resistance could be a reflection of some of those factors relating to selectively vulnerability already discussed above. A further unique property of muscles spindles that may affect their relative vulnerability/resistance to pathological stimuli is the capsule that surrounds the muscle spindle (see section 1.4.3). This capsule will create a unique microenvironment surrounding IA/II sensory and most γ -motor nerve terminals that may help buffer against some external stimuli (Barker 1974) and perhaps precondition these nerve terminals against hypoxic injury. Overall, further studies are needed to address the relative resistance of IA/II sensory and γ motor nerve terminals to hypoxia-reperfusion injury to confirm/disprove the preliminary results presented here. These studies should investigate if IA/II and γ -motor nerve terminals are vulnerable to more intense and/or chronic hypoxia/ischaemic-reperfusion injuries as other studies indicate that they are not invulnerable to ischaemia (Cipollone 1898; Diwan and Milburn 1986). These studies will need to address the problem of the generally poor immunohistochemical staining of muscles spindles that hindered analysis of any potential pathological response(s) here. This will probably be best achieved with quantitative electron microscopy, where responses of IA, II, and γ -nerve terminals can be assessed without the complication of poor penetration of antibodies and the lack of postsynaptic staining needed for comparative analysis. Once such a protocol is in place, the hypoxia-reperfusion model system here provides the opportunity to complete a temporal analysis of the possible responses of sensory and motor nerve terminals of the muscle spindle where other studies have indicated different vulnerabilities following *in vivo* ischaemia (Cipollone 1898; Diwan and Milburn 1986), alongside that of α -motor nerve terminals. Such studies may also help to provide valuable insights into the factors that govern selective vulnerability/resistance between, as well as within, groups of neurons.

3.4.5 Summary

I have provided further evidence to show that hypoxia-reperfusion injury triggers rapid and selective disassembly of α -motor nerve terminals that includes functional failure. The extent of pathology appears linked to age and fibre type but why different α -motor nerve terminals from different skeletal muscle fibres show selective vulnerability/resistance to hypoxia-reperfusion injury (and other

pathological stimuli are still unknown) but is likely to involve a number of complex variables. I have also shown that the neuroprotective *Wld^s* mutation fails to protect α -motor nerve terminals against hypoxia-reperfusion injury. This may suggest that despite some morphological evidence, the molecular pathways of Wallerian degeneration are not involved in hypoxia-reperfusion injury. Alternatively, it may also suggest that the mechanism of *Wld^s* mediated neuroprotection is upstream of direct inhibition of aerobic respiration or that the neuroprotective mechanism of *Wld^s* was bypassed. Collectively, this may indicate that the molecular pathways of Wallerian degeneration may still have a role to play in α -motor nerve terminal disassembly in response to hypoxia-reperfusion injury. Further work is required to understand the underlying causes of selective vulnerability/resistance, the mechanisms of α -motor nerve terminal disassembly and how these different mechanisms relate to each other (i.e., are they completely independent or do they exist on a mechanistic continuum?), and how the *Wld^s* mutation confers protection. The model system described here may provide an ideal tool to investigate some of these possibilities.

Chapter 4

A pilot study examining the response of α -motor nerve terminals to hypoxia in real time

4.1 Introduction

In the mid 1990's it was discovered that the gene for green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* (first characterised by Shimomura *et al.* 1962) could be successfully expressed in other species and that the chromophore could autofluoresce upon excitation with blue light to produce a fluorescent signal without apparent toxicity (Chalfie *et al.* 1994; Inouye and Tsuji 1994b). More recent advances in molecular genetics have shown that endogenously expressed GFP (27kDa: Heim *et al.* 1994) and related colour-shift mutants (XFPs) can be used in a range of cells including whole animals to specifically identify chosen macromolecules, sub-cellular compartments or tissues (Cubitt *et al.* 1995; Tsien 1998; Niell and Smith 2004; Misgeld and Kerschensteiner 2006). On the genetic background of the C57Bl/6 mice, Feng *et al.* (2000) generated several strains of transgenic mice that selectively express enhanced yellow fluorescence protein (YFP) under the neuron specific regulatory elements of the Thy1.2 immunoglobulin gene (Caroni 1997a; Lichtman and Sanes 2003). These transgenic mice express YFP in the cytoplasm (Misgeld and Kerschensteiner 2006; Walsh and Quigley 2008) of the majority of their neurons, including α -motor neurons and can be visualised using a standard epifluorescence microscope fitted with FITC filter sets. YFP also has suitable emission spectra to be utilised with red light emitting fluorophores such as TRITC for dual labelling and is still excitable after formaldehyde fixation (Chalfie *et al.* 1994; Feng *et al.* 2000). These desirable characteristics have meant that these mice and related genetic strains are now in routine laboratory use, in many cases replacing more time consuming and irksome histochemical methods that have been traditionally used to examine neuron morphology (Bridge *et al.* 2009). One particular advantage of these transgenic mice is the ability to examine neuron morphology over time *in vivo* or in *ex vivo* explants to provide valuable information on dynamic responses that otherwise would be impossible to obtain by studying static images (Feng *et al.* 2000; Lichtman and Fraser 2001; Lichtman and Sanes 2003; Niell and Smith 2004; Misgeld and Kerschensteiner 2006). The potential for live-imaging techniques to give unprecedented spatiotemporal data is exemplified by results obtained by repeated imaging of dorsal root ganglia (DRG) by Kerschensteiner *et al.* (2005). Within minutes of axotomy, symmetrical axonal swelling was rapidly followed by localised retraction (200 to 300 μ m) and fragmentation of the proximal and distal DRG nerve stumps. This 'acute axonal degeneration' occurred within 5min and accounted for 90% of all axonal loss over a 4hr period. These previously unknown events following axotomy preceded a static phase lasting 6-24hr in the proximal nerve stump and ~30hr in the distal nerve stump before the onset of regenerative and degenerative (classic Wallerian) processes respectively. Similar imaging studies during developmental synapse elimination of α -motor nerve terminals also provided novel results, describing the shedding of membrane bound fragments or 'axosomes' from withdrawing α -motor nerve terminals that were engulfed by Schwann cells (Bishop *et al.* 2004). A study examining the effects of dendritic spine morphology during and following ischaemia-reperfusion injuries have shown that during mild

ischaemia spines remain stable but 10-30mins after the onset of severe ischaemia spines become swollen and lost. During reperfusion some spines can recover within 60mins but most will later degenerate by 24hr (Zhang *et al.* 2005). These transgenic XFP lines of mice have also been used to examine tourniquet induced ischaemia-reperfusion injury at α -motor nerve terminals (David *et al.* 2007) but this study did not explore the possibility of using these mice to examine the responses of α -motor nerve terminals in real time by repeated imaging. To provide further details of the potentially dynamic, spatiotemporal responses of α -motor nerve terminals to these types of injuries, the following chapter describes the results obtained in a pilot study looking at the response of mouse α -motor nerve terminals to hypoxia by repeated/live imaging in *ex vivo* TA muscles.

4.2 Methods

4.2.1 Tissues

Adult (8-14wk old) Thy-1/16-YFP C57Bl/6 mice (YFP mice) were obtained from Jackson Laboratories, USA (genetic strain created by Feng *et al.* 2000) and maintained at the University of Leeds. TA muscles were dissected as described in Chapter 3 but after preparation was split up the midline, the two hemi-preparations were trimmed to leave 2x ~2.5cm² pieces of tissue with the window of intact TA muscle fibres in the centre (see Fig 4.1 and Fig3.1 for details). The better of the two preparations, determined by muscle fibre integrity, density of residual overlying tissue and fat deposits was selected for live imaging. *N*= number of animals, *n* = number of muscles.

4.2.2 Tissue processing and live imaging system

TA muscles preparations had their postsynaptic endplates visualised with TRITC conjugated α -bungarotoxin (BTX) for 10mins (5 μ g/ml in Kerbs' solution: Invitrogen) that directly labels AChRs followed by a brief rinse with fresh HEPES buffered Krebs' solution (see Chapter 2 for details). TA muscle preparations were then pinned out under tension in 3ml petri dishes lined with Sylgard and mounted into a microperfusion chamber (Harvard Instruments: see Fig 4.1) loaded on to the stage of the upright epifluorescence microscope (Zeiss Standard 10) in a darkened room to reduce light exposure. The microscope was fitted with FITC/TRITC filter sets, a 50W mercury vapour light

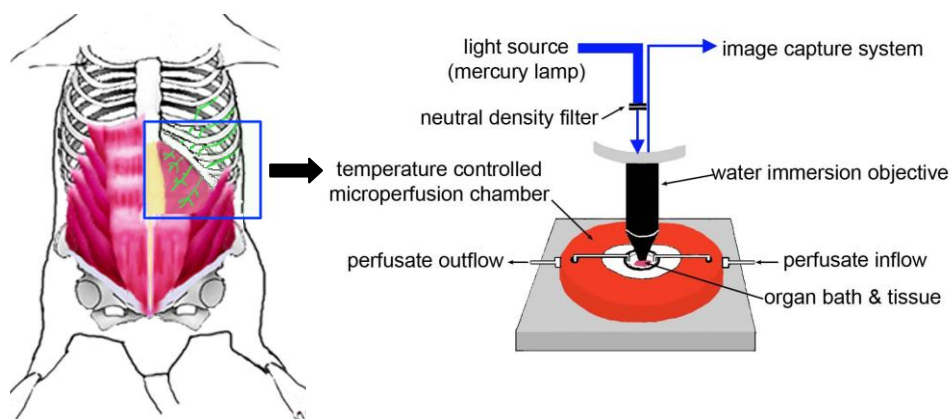


Fig 4.1 Schematic overview of the live imaging set-up. TA muscles from adult YFP transgenic mice were dissected free from surrounding tissues and trimmed to a size corresponding to the blue-boxed area (LEFT). These preparations were then placed in an organ bath/superperfusion chamber mounted onto the stage of an upright epifluorescence microscope (RIGHT).

source, x50 Zeiss water immersion lens (0.8 NA) with the addition of a neutral density filter (No 6) to reduced illumination intensity by 94%. Images of the nerve terminals and postsynaptic endplates were captured over time using a monochrome Cohu 4910 CCD camera (Vista Vision, Steventon, UK) using Scion Image capture software (Maryland, USA) running on an Apple Macintosh Quadra 650 microcomputer. Each image was averaged, typically between 50-100 frames with a total light exposure time of 3-5s per image. In all instances the postsynaptic endplate was only imaged at the beginning and end of the experiment, while the nerve terminal was imaged at indicated intervals and the light path completely blocked in-between each exposure to help reduce overall light exposure. The TA muscle preparations were maintained by superperfusion performed by gravity flow of Krebs' solution into the base of the chamber and vacuum aspiration at the top of the chamber to create a flow across the preparation. Chamber volume was maintained at ~2ml (flow rate 1.6ml per min, total saline exchange every to 2-3min) and temperature was maintained at 22-25°C. In control experiments, TA preparations were superperfused with Krebs' solution sparged with 95%:5% O₂:CO₂ gas or pressurised air. In hypoxic experiments the Krebs' solution was sparged with 100% N₂ gas. In a subset of experiments, TA preparation were maintained in ~1ml static volume of Krebs' solution with α -latrotoxin (LTX: Alomone labs, UK) at a final dilution of 1nM (the upper limit of the suggested working concentration from the supplied data sheet). In a different set of experiments, lumbrical, TA and TS muscles (see Chapters 2 and 3 for details) were fixed with 4% formaldehyde in PBS for 30min immediately after dissection and incubation in BTX (i.e., no live imaging was carried out). After 3x15min wash in PBS muscles were mounted onto slides as described previously.

4.2.3 Image processing

Images were aligned in Scion Image for Windows (release 4) and reconstructed in Adobe Photoshop CS2 with each image adjusted for brightness, contrast and coloured as required. Each of the live imaging sequences was animated in Adobe ImageReady CS2 and converted to a high resolution QuickTime Movies that can be seen using RealNetworks, Inc free version of RealPlayer for Mac and PC (www.real.com). These movies are supplied on enclosed DVD.

4.3 Results

4.3.1 Characteristics of α -nerve terminals and axons from YFP mice

As the colony of YFP-mice maintained at the University of Leeds was newly established, initial observations examined the expression of YFP in nerve terminals from fixed lumbrical, TA and TS muscles (i.e., without live imaging). This showed that YFP was expressed in all the α -motor neurons and nerve terminals supplying these three muscle groups and when postsynaptic AChRs were labelled with BTX, YFP could be seen to fully oppose BTX staining as shown previously (Feng *et al.* 2000). There was also a low level incidence of synaptic guttering, where small regions of postsynaptic BTX could be observed with no opposing presynaptic YFP (Fig 4.2A). This is generally accepted as being the result of ongoing synaptic reorganisation (Cardasis and Padykula 1981; Wernig *et al.* 1984; Wernig and Herrera 1986; Lichtman *et al.* 1987; Wigston 1989; 1990) and appears to correspond to no more than 1 or 2 synaptic boutons, affecting less than an estimated 1% of the total population of nerve terminals. The pre-terminal axons in these transgenic YFP mice however, were nearly always observed to be highly distended or extremely thinned, vacuolated and with evidence of a heterogeneous distribution of YFP (Fig4.2B). The severity of these features appeared greater in lumbrical muscles, becoming less severe in axons located in the intramuscular nerve and axons in TA and TS muscle (Fig 4.2C). On occasion α -motor nerve terminals in the lumbrical muscles were observed to have sprouts (Fig4.2D), a feature that I have never observed in wild-type animals or very rarely by others (Wernig *et al.* 1984; Wernig and Herrera 1986; Lichtman *et al.* 1987; Parson *et al.* group, unpublished observations (c.f Barker and Ip 1966)). Although not formally assessed, these animals showed no signs of behavioural/functional impairment compared to their wild-type relatives and bred well until 5 months of age when they were culled.

4.3.2 Live imaging of Nerve Terminals under control conditions

A live imaging system was developed (see methods) in which the nerve terminals of *ex vivo* TA could be observed over time and that could be used to examine α -motor nerve terminal responses to hypoxia. As these preparations have minimal distal nerve stumps (<0.3cm), live imaging was limited to 3hr. Overall, the success rate of this system was low, primarily due to the requirement of nerve terminals to be and remain in the plane of view for 3hrs. Even minor drifts or changes in muscle fibre tension (in some cases possibly due to spontaneous minor muscle fibre contractures) could cause changes in α -motor nerve terminal orientation so they were no longer focused on a single plane of view and/or to have shifted away from the plane of view.

In control conditions, nerve terminals were imaged every 15min over a period of 3hr to show that overall, the gross morphology of the nerve terminals, endplate and muscle fibre remained stable when perfused with Krebs' solution sparged with 95%:5% O₂:CO₂ ($N=3$, $n=3$) or pressurised air ($N=3$,

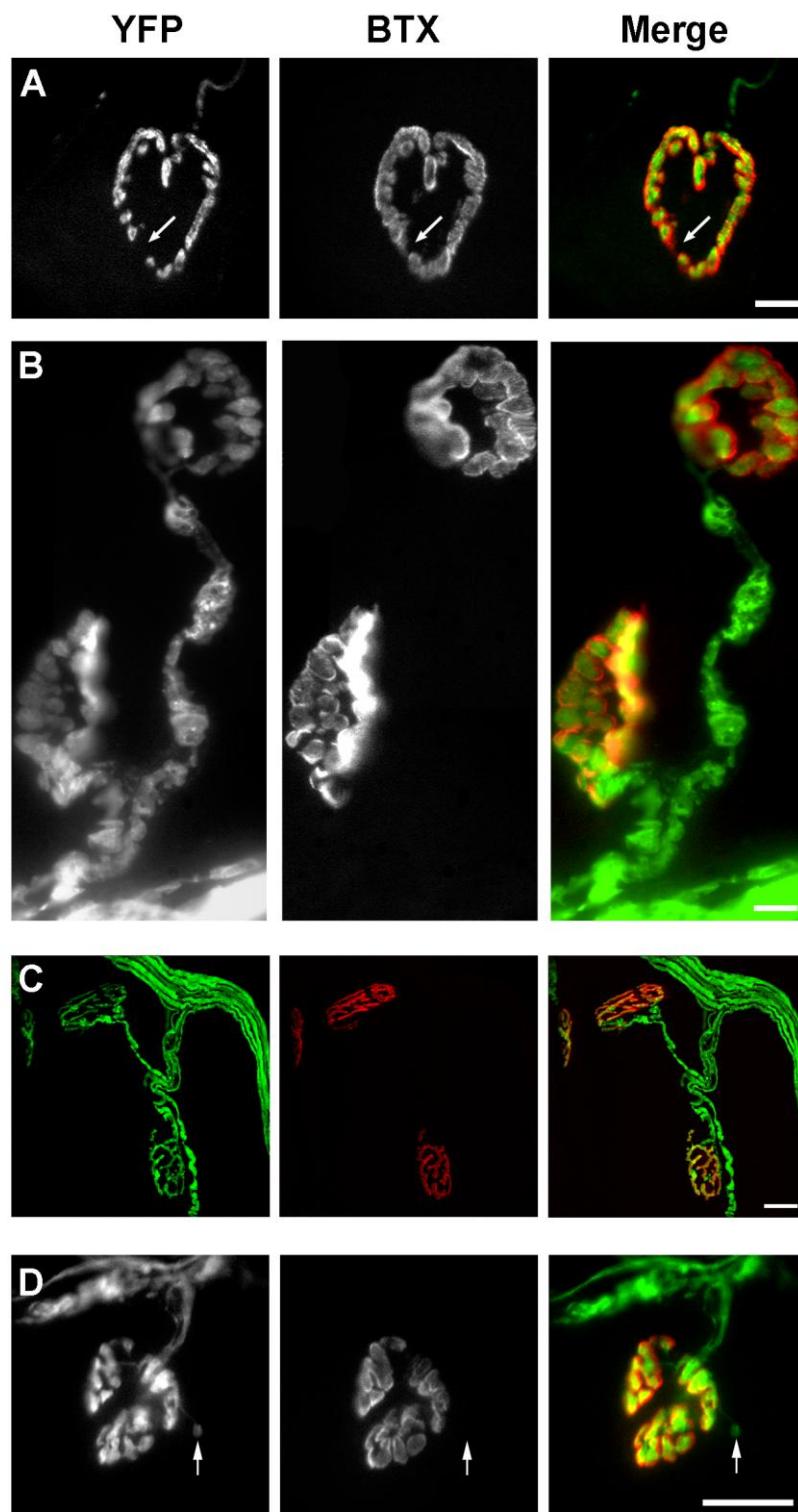


Fig 4.2 α -motor nerve terminals and axons from YFP transgenic mice have a basal level of pathology. While most α -motor nerve terminals visualised by endogenously expressed YFP (GREEN in merged images) fully oppose their postsynaptic endplates visualised with BTX (RED in merged images), there was evidence of synaptic gutting like that seen in (**A**: arrow) from a lumbrical muscle. The level of synaptic gutting was similar to that observed to occur in wild-type animals and is generally thought to present on-going re-organisation. Pre-terminal axons in YFP mice were observed to be highly distended and/or thinned with a heterogeneous distribution of YFP and vacuoles. This affected lumbrical muscles (**B**) more than TA (**C**) or TS muscles, and was less apparent in the larger intramuscular nerve branches (**C**). Although rare, α -motor nerve terminals from these mice were also occasionally observed to have sprouts extending away from the terminal arborisation, either with (**D**: from lumbrical muscles) or without opposing BTX staining. A, B, and D are micrographs, C is a confocal projection (see Chapter 2 for details). Scale bars A&B 10 μ m, C&D 25 μ m.

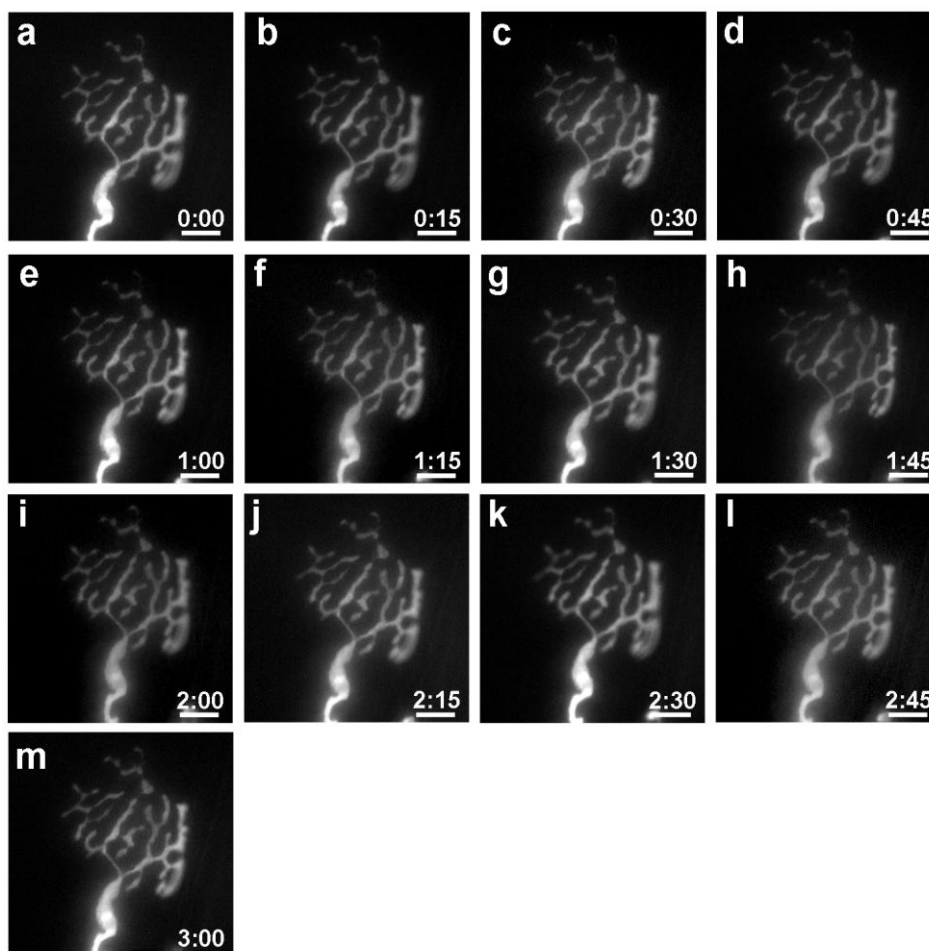
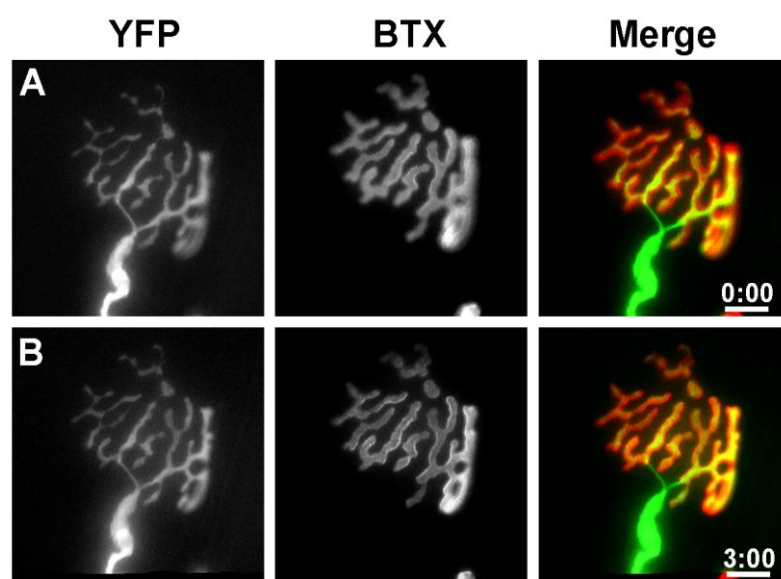


Fig 4.3 α -motor nerve terminals repeatedly imaged in control conditions did not typically show any changes in morphology. TA muscles from YFP transgenic mice were maintained in a superfusion chamber mounted onto the stage of a microscope. In control conditions using HEPES buffered Krebs' solution that was sparged with 95%:5% O₂:CO₂ or pressurised air (shown), α -motor nerve terminals (YFP, GREEN in merged images) were imaged every 15min for 3hr and compared to their postsynaptic endplates (visualised with BTX: RED in merged images) that were only imaged at the beginning and the end of the experiment. Comparison of presynaptic YFP and postsynaptic endplate morphology at the beginning (**A**) and the end of the experiment (**B**) shows no apparent changes in morphology. Serial reconstruction of images of the terminal (**a-m**) with time shown in hr:min in the lower left of each image also show no apparent changes in morphology. Scale bar 10 μ m

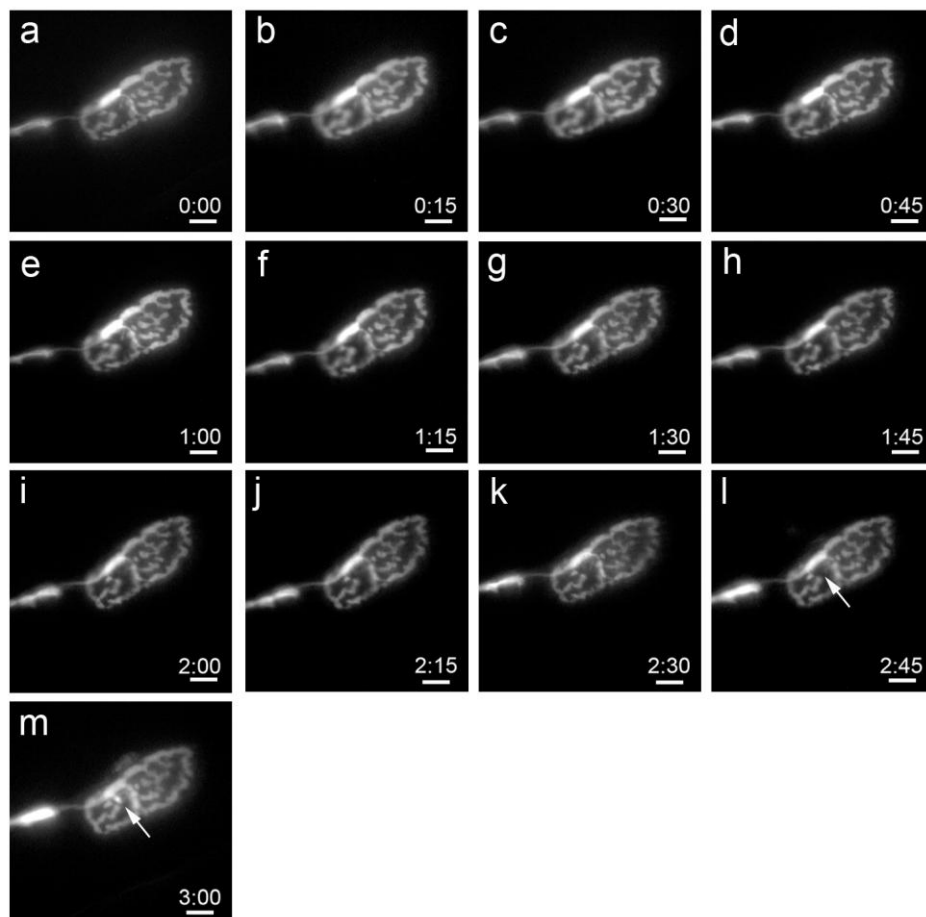
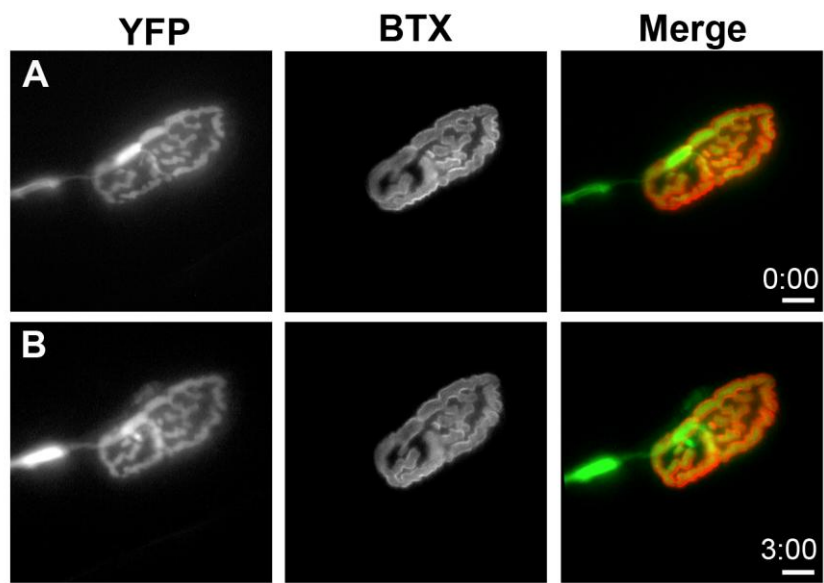


Fig 4.4 Minor changes in morphology could be observed in repeatedly imaged α -motor nerve terminals in control conditions. Presynaptic YFP (GREEN in merged images) and postsynaptic endplates (visualised with BTX: RED in merged images) is compared at the beginning of the experiment (**A**) and after 3hr (**B**) where the α -motor nerve terminal had been imaged every 15mins (**a-m**) while being superfused with HEPES Krebs' solution sparged with pressurised air. α -motor nerve terminal morphology remained unchanged until between 2hr 45min and 3hr where there was a sprout-like projection (l&m: arrow). There was no apparent change in postsynaptic endplate morphology and the sprout-like projection was not opposed by BTX staining. Note that the sprout-like projection comes from a region of the nerve terminal arborisation that appears with intense YFP fluorescence. Time in the lower left of images hr:min, scale bar 10 μ m.

$n=3$:Fig 4.3). The reason why air instead of 95%:5% $O_2:CO_2$ was used in some experiments was because there were increasing concerns that the high O_2 concentration may be causing ROS formation as part of the mechanism of phototoxicity (see below and discussion). In general, however no difference was noted between those preparations sparged with air or those with 95%:5% $O_2:CO_2$. Close examination of the serial reconstructions over time however, did demonstrate that minor alterations in morphology of the nerve terminal could occur even in control experiments (Fig 4.4). In one control experiment sparged with air, an outgrowth, 'sprout-like' projection can be seen to develop at 2hr45min. In another control experiment that was sparged with 95%:5% $O_2:CO_2$, no change in morphology occurred but there were changes in the distribution of YFP, with regions appear more or less bright. In this case it was difficult to determine if this reflected real changes in YFP or if it was an artefact related to minor changes in α -motor nerve terminal orientation and/or focal plane.

4.3.3 Live imaging of Nerve Terminals under hypoxic conditions

To investigate the response of nerve terminals from TA muscles to hypoxia, the Krebs' solution perfusing the TA muscles was replaced with a solution that had been sparged with 100% N_2 gas ($N=3$, $n=3$:Fig 4.5). No significant changes were observed in the postsynaptic endplates or muscle fibres over the 3hr experiment. In α -motor nerve terminals, serial reconstructions showed a range of small but rapid and dynamic morphological events prior to the sudden loss of YFP intensity that included swelling and rapidly appearing/disappearing sprouts and small fragments that were typified by events shown in Fig 4.5. In this α - nerve terminal, these small morphological events could be observed within 15mins until 2hr 30min where there was a sudden loss of YFP intensity between 2hr 30min and 2hr 45min with the nerve terminal appearing almost un-identifiable against background autofluorescence. When this nerve terminal (and those in other similar experiments) was imaged at the end of experiment without the neutral density filter (i.e., incident light level was returned to 100% rather than 6% used during the live imaging process), the nerve terminal appeared to still be largely present but with a highly punctate, ghostly morphology. At the end of the experiment, examination of α -motor nerve terminals that had not been in the field of illumination appeared normal, with no apparent perturbations in morphology. Very similar results were also obtained from α -motor nerve terminals that were superperfused with Krebs' solution that had previously been sparged with 95%:5% $O_2:CO_2$ gas but where sparging had been stopped at the onset of imaging ($N=2$, $n=2$). These results clearly show that nerve terminals can have a highly dynamic and rapid response to pathological insults but indicate that hypoxia and live imaging protocol of YFP filled neurons were probably acting as co-stimuli to induce the observed α -motor nerve terminal pathology.

4.3.4 Live imaging of nerve terminals following application of α -latrotoxin (LTX)

To try and help understand the morphological events observed in nerve terminals described above, nerve terminals were imaged in the 1ml static volume of oxygenated Krebs' solution containing 1nM of α -latrotoxin (LTX). LTX is a neurotoxin selective for nerve terminals and is known to cause rapid synaptic vesicle release, swelling and lysis of motor nerve terminals (Clark *et al.* 1970; Okamoto *et al.* 1971; Duchen *et al.* 1981; O'Hanlon *et al.* 2003; Ushkaryov *et al.* 2004). All but one of the attempts to live image the response of nerve terminals to LTX in TA muscles was unsuccessful due to significant muscle fibre contractures. In one experiment, (Fig 4.6) contractures were suitably inhibited to allow for continuous live imaging by increasing the incubation time of TA muscles preparation in FITC conjugated BTX to 30min. BTX is highly selective, irreversible ligand at postsynaptic AChRs and can cause neuromuscular blockade by preventing muscle fibre depolarisation in response to synaptic vesicle release (Chang 1999). Imaged every 5min (0hr20min time point was missed), the α -motor nerve terminal incubated in LTX remained unchanged for 40min, following which there was rapid loss of several nerve terminal boutons by 45mins. Other regions of the arborisation were lost between 1hr 10mins and 1hr 15mins leaving behind ghostly and punctate YFP fluorescence, like that observed affecting the whole α -motor nerve terminal during hypoxia. Several swellings also appeared at 1hr and 1hr 25min like those observed during the hypoxia experiment, however these swelling remained largely unchanged until the end of the LTX imaging at 1hr 30min. Observations of the morphology in α -motor nerve terminal that had not been repeatedly imaged at the end of the experiment also showed some pathological changes, i.e., loss of part or all of overlying terminal as determined by YFP. This suggests that there are some similarities between the response of α -motor nerve terminals to LTX and hypoxia when they are repeatedly imaged.

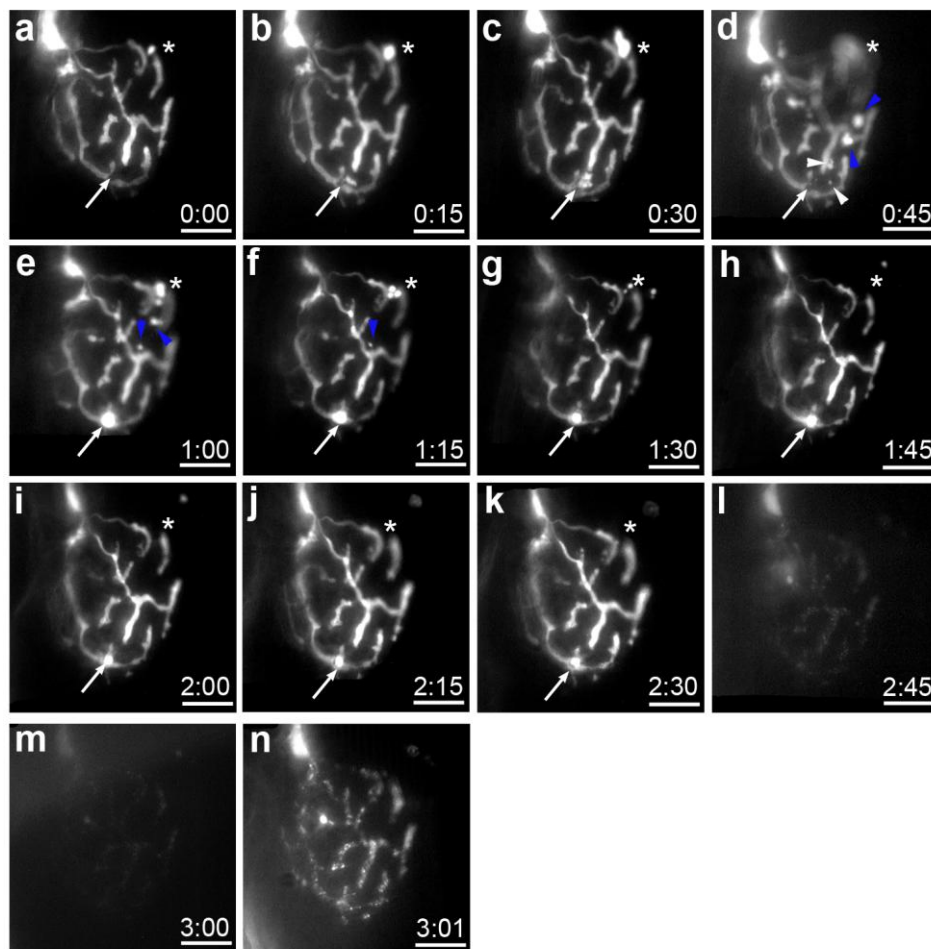
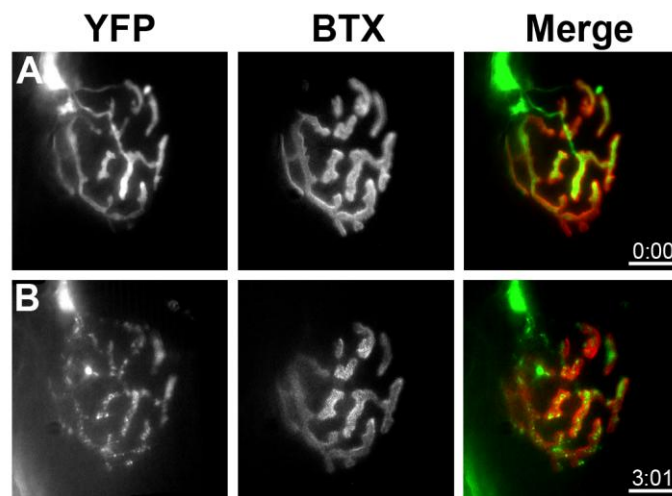


Fig 4.5 Rapid and dynamic changes in morphology could be observed in repeatedly imaged α -motor nerve terminals in hypoxic conditions. Presynaptic YFP (GREEN in merged images) and postsynaptic endplates (visualised with BTX: RED in merged images) is compared at the beginning of the experiment (**A**) and after 3hr (**B**) where the α -motor nerve terminal had been imaged every 15mins (**a-m**) while being superfused with HEPES Krebs' solution sparged with N₂ gas. Changes in α -motor nerve terminal morphology could be observed within 15min (b) with a sprout-like event (arrow) that appeared to swell by 30min (c) and to have disappeared by 45min (d) but surrounded by small fragments (white arrowheads). The region of the terminal arborisation from which the sprout-like projection arose appeared with average intensity YFP but following the loss the sprout-like projection, this region becomes very YFP intense and possibly swollen that is still visible by 2hr: 30min (arrows). Also beginning within 15min after the onset of hypoxia and imaging, a range of changes are observed in the region marked by the asterisk. Already quite YFP intense at the beginning of the experiment, this region begins to swell for 30min but in (d: 45min) this region becomes obscured even though the rest of the nerve terminal remains in relatively clear focus. This perhaps represents release of YFP from the nerve terminal. In the next image (e:1hr:0min) this region and the YFP-intense swelling is visible but the swelling appears reduced (compare c and e). This swelling then appears to fragment by 1hr:15min (f) and the fragments appear to move away from nerve terminal or disappear over the next 45min (images g-k). Various other fragmentation and swelling events are indicated by blue arrowheads. At 2hr:30min (k) the α -motor nerve terminal still appears with good YFP intensity but by 2hr:45min (l) there is dramatic and synchronous loss of YFP from the entire arborisation leaving a 'ghostly' shadow of the nerve terminal by 3hr:0min (m). By removing the neutral density filter and returning incident light levels to 100% (n) the α -motor nerve terminal is again visible and appears to fully oppose its postsynaptic endplate (B) but YFP fluorescence has become highly punctate (m&B). Time in the lower left of images hr:min, scale bar 10 μ m.

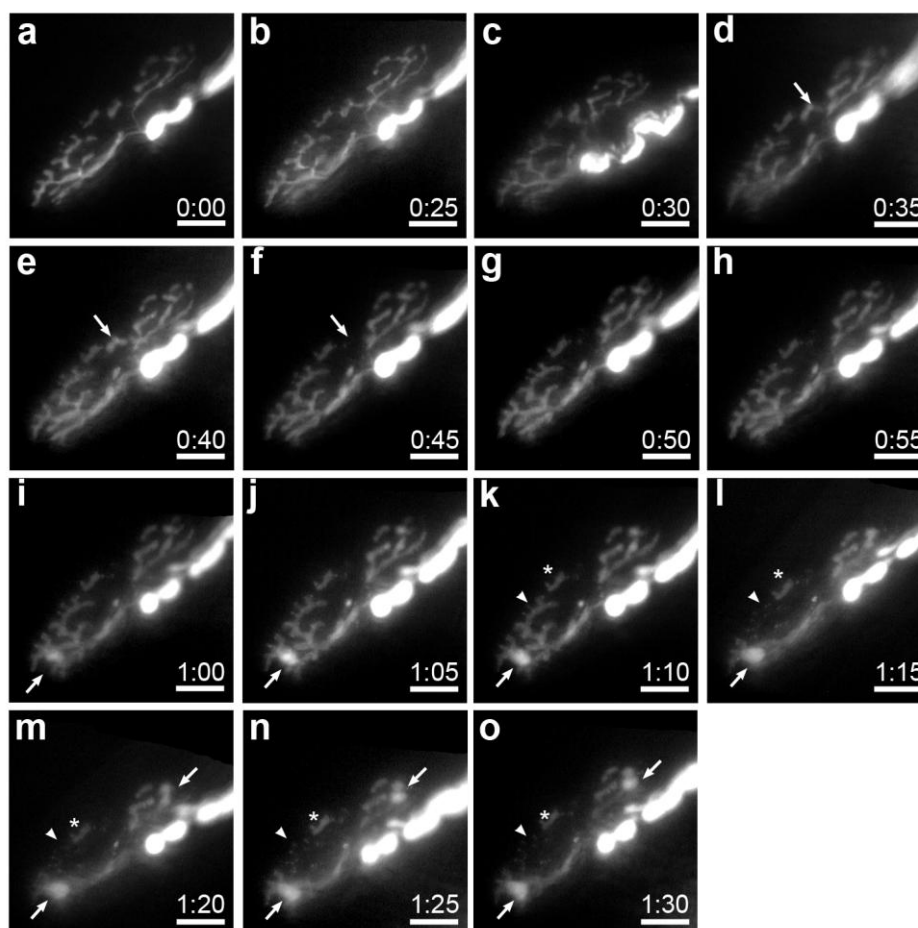
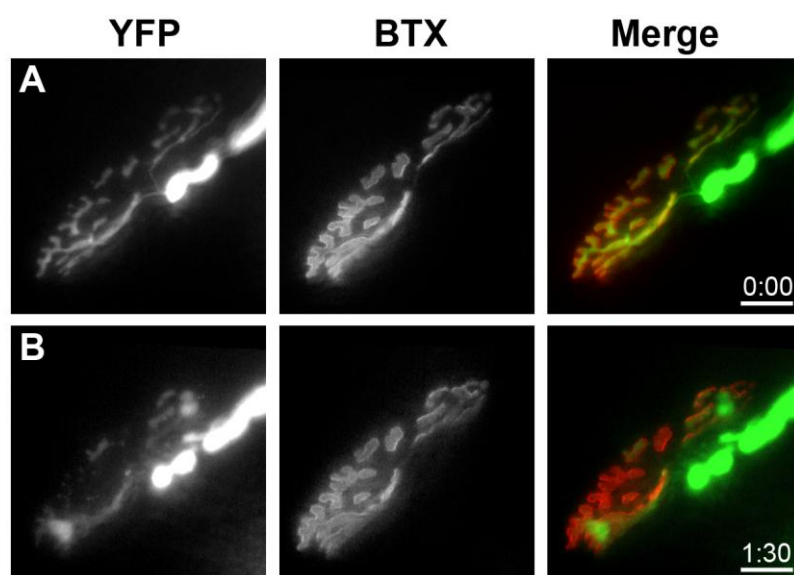


Fig 4.6 Observations of the changes in α -motor nerve terminal morphology in response to LTX shows some similarities to that observed during hypoxia.

Presynaptic YFP (GREEN in merged images) and postsynaptic endplates (visualised with BTX: RED in merged images) is compared at the beginning of the experiment (**A**) and after 1hr30 (**B**) where the α -motor nerve terminal had been imaged every 5mins (**a-o**) while being maintained in a static volume of oxygenated HEPES Krebs' solution with 1nM of α -latrotoxin (LTX). In the presence of LTX there was no observable change in α -motor nerve terminal morphology for the first 35min (images from 5-20min have been omitted) but there were some weak muscle fibre contractures (compare overall morphology in c with previous images). Between 40min (e) and 45min (f) there is complete loss of YFP from a small region corresponding to one or two synaptic boutons (arrow). No further changes were noted until 1hr (i) after on the onset of imaging and application of LTX, where a small region of the nerve terminal appears to swell (arrow). This swelling, that is similar to some of the events observed during the hypoxia experiments, remains relatively unchanged until the end of the experiment at 1hr:30min (o). Between 1hr:10min (l) and 1hr:15min there is loss of another, larger region of the nerve terminal arborisation, leaving behind a ghostly punctate shadow (arrow), again similar to some of the events observed during the hypoxia experiments. Loss of this region leaves another region (corresponding to one or two synaptic boutons) apparently isolated from the rest of the arborisation (asterisk) which remains unchanged for the duration of the experiment. Another swelling appears at 1hr:20min (m: upper most arrow) and appears to increase in size until 1hr:30min (o). Time in the lower left of images hr:min, scale bar 10 μ m

4.4 Discussion

The results presented here show that α -motor nerve terminals from transgenic mice that express endogenous YFP in their neurons can undergo a very rapid and dynamic response to pathological stimuli. These results however raise concerns about the consequences of expressing fluorescent proteins within neurons and also the ability to monitor their responses to hypoxia in real time.

4.4.1 Axonal and α -motor nerve terminal pathology induced by YFP

YFP is a colour-shift mutant of GFP created by substitution of Thr²⁰³ with aromatic amino acids that results in a subtle change in emission spectra and its yellowish appearance (Tsien 1998). Due to the fact that GFP and its colour-shift mutant relatives (XFPs) are based on a naturally occurring protein, they are generally considered to be innocuous and have been successfully expressed in a whole range of cell types to identify cells, sub-cellular compartments and macromolecules (Cubitt *et al.* 1995; Tsien 1998). Examination of tissue from YFP-mice that had not undergone live imaging showed that the axons of these mice were highly distended and vacuolated with regions of extreme thinning and very heterogeneous distribution of YFP. There was also a low-level incidence of α -motor nerve terminal pathology with outgrowth or sprouting events that are relatively rarely seen in control, wild-type animals (Wernig *et al.* 1984; Wernig and Herrera 1986; Lichtman *et al.* 1987 (c.f., Barker and Ip 1966¹⁰)). Similar axonal pathology including localised axonal swelling and thinning but not as severe as that described here, could also be observed in the same YFP strain of mice but a different colony of mice maintained at the University of Edinburgh (personal observations and Gillingwater *et al.* group, unpublished observations). In reviewing the original publication that described this strain of transgenic mouse, there was some evidence of unusual axonal swelling in their micrographs (Feng *et al.* 2000). The underlying cause of this is unclear however, the creators of these mice did comment on the high variability of Thy1.2-YFP gene insertions site and copy number. It would seem the most likely explanation for these differences would be that the two colonies of YFP-mice may have differing expression levels of YFP due to differing genetic backgrounds (the colony maintained at Edinburgh University is known to have been out-crossed and therefore may have lower YFP expression levels: Derek Thompson, personal communication). High levels of YFP protein are known to cause dimerisation leading to oligomerisations (Cubitt *et al.* 1995; Shaner *et al.* 2005) and excess levels of YFP-protein aggregates may account for distension and swelling of axons observed here.

¹⁰ In this study the authors found a high degree of sprouting from three species than on average effected a total of ~25% of α -motor nerve terminals. Most of these sprouting events were of the nodal and pre terminal varieties but the observed frequency of terminal sprouts like that observed and discussed here (referred to as ultraterminal by Barker and Ip(1966)) was low. None were observed in muscles from the cat or the rabbit, and only 3 instances out of the 37 groups of α -motor nerve terminals were observed in the rat. This frequency is still higher than that described by others (Wernig *et al.* 1984; Wernig and Herrera 1986; Lichtman *et al.* 1987) but the rat was the species least studied by Barker and Ip (1966) and there is no clear indication of numbers (or clear age) of rats used in their study. It may therefore be possible that this higher frequency of ultraterminal sprouting found in rats in this study was due to naturally occurring low-frequency pathological responses and/or perhaps due to using young animals where the dynamic morphological events of the process of developmental synapse elimination have yet to be fully completed. This in combination with a potentially low number of repeats may perhaps resulted in a higher percentage of ultraterminal sprouts.

The reason(s) for the apparent difference in these pathological features between axons located in the intramuscular nerve and pre-terminal axons of α -motor nerve terminals innervating TA and TS (less affected) and those from pre-terminal axons and from the lumbrical muscles (more affected) is unknown. It is also unknown and highly surprising that despite the pathological appearance of axon and nerve terminals that behaviour and breeding, in these YFP-mice was no different from their wild-type litter mates/counterparts. These observations of pathology in YFP-transgenic mice are consistent with a recent report of pathology in CNS neurons from the related YFP-H mouse strain that only express YFP in a random subset of neurons (Bridge *et al.* 2009). These authors report a significant increase in number of swellings or spheroids in the gracile tract, gracile nucleus and dorsal root ganglia in YFP-H mice that was independent of the effects of ageing. They show that up to 16 months of age no behavioural changes could be observed despite increasing frequency of axonal swelling. They also note that number of swellings increases in mice homozygous for YFP compared to heterozygous animals to indicate that increased expression of YFP is the cause of pathology. Swelling, with or without vacuolisation is a common feature of CNS ageing process (Yoshikawa *et al.* 1985) but is also related to pathological and neurodegenerative disease stimuli including Alzheimer's disease and other *tau*-pathies (Lewis *et al.* 2000; Brendza *et al.* 2003; Tsai *et al.* 2004), Parkinson's disease (Galvin *et al.* 1999), stroke (Dewar *et al.* 1999; Lipton 1999), ALS (Tu *et al.* 1996), Creutzfeldt–Jakob disease (Liberski and Budka 1999), hereditary spastic paraplegia (Ferreirinha *et al.* 2004), Niemann-Pick disease (Elleder *et al.* 1985; Bu *et al.* 2002), multiple sclerosis (Ferguson *et al.* 1997), gracile axonal dystrophy and others (Mi *et al.* 2005). In general, axonal swelling is very rare in the PNS but in the CNS, it is thought to cause disruption of axonal transport and precede axonal degeneration (Mi *et al.* 2005). There are also increasing numbers of reports of deleterious consequences of XFP in other systems. There have been several reports of the difficulty in generating stable XFP cell lines, with cells undergoing cytotoxic apoptosis 48hr to 96hr after transfection (Lamhonwah and Tein 1999; Liu *et al.* 1999; Shaner *et al.* 2005). XFP expressed in myoblasts caused a 5-fold increase in cytokine production (Mak *et al.* 2007) and impairment of contractile function due to XFP interaction with myosin (Agbulut *et al.* 2007). This is the likely cause of impaired left ventricular ejection rates in rats (Agbulut *et al.* 2006) and may be responsible for an independent report of GFP induced cardiomyopathy (Huang *et al.* 2000). XFPs have also been shown to reduce mouse embryonic viability in an expression level dependant manner (Devgan *et al.* 2004), alter ubiquitination pathways that are involved in protein degradation *in vitro* and *in vivo* (Baens *et al.* 2006), sensitise neuroblastoma cell lines to cytotoxic and chemotherapeutic agents (Goto *et al.* 2003), and to be neurotoxic to dopaminergic neurons (Klein *et al.* 2006). Co-expression of XFP with β -galactosidase, that alone is not neurotoxic, caused developmental abnormalities with apoptosis of mouse forebrain neurons and death by 4 week of age (Krestel *et al.* 2004). When XFP was co-expressed with members of Bcl-2 family, mitochondria become aggregated and later triggered apoptosis (Aokage *et al.* 2004) while XFP fused to DNA has been shown to cause production of anti-DNA antibodies (Moens *et al.* 2002). It is unclear how and why XFPs have these effects but XFP aggregation has been suggested (Shaner *et al.* 2005) as well as the formation of ROS (Greenbaum *et*

al. 2000; Goto *et al.* 2003) with further indications that XFPs produce H_2O_2 (a ROS) as part of their fluorescent mechanism and probably in a 1:1 ratio (Heim *et al.* 1994; Inouye and Tsuji 1994a; Tsien 1998). Production of ROS, particularly in highly expressing tissue may easily overwhelm endogenous protective mechanisms to cause irrecoverable damage to macromolecular structures, enzymes, cell membranes and to possibly trigger cell death (Halliwell 2006). In the results present here, while there was evidence of synaptic guttering that would indicate loss of terminal boutons, this was at a level consistent with wild-type controls. I did not observe any evidence of degenerating axons or nerve terminals that parallels the finding from the CNS of YFP-H mice (Bridge *et al.* 2009). The low levels of sprouting events observed at the nerve terminal may however indicate that there are some functional deficits as sprouting is known to be caused by partial denervation (Lichtman *et al.* 1987; Tam and Gordon 2003) and conduction block such as that produced by botulinum toxin (Botox: Duchen and Strich 1968; Duchen 1970; de Paiva *et al.* 1999; Meunier *et al.* 2002; Rogozhin *et al.* 2008) or cholinesterase inhibition (Kawabuchi *et al.* 1991). Overall, these results suggest that while expression of YFP in α -motor neurons is not toxic, it is certainly not innocuous and maybe increasing basal stress levels due to overly high YFP expression, aggregation and/or ROS formation. This therefore raises concerns about subjecting YFP neurons to additional pathological insults, particularly those types of stimuli linked with ROS formation such as hypoxia. It may also raise concerns about novel findings in studies that utilise these mice. For example prior to the current study, the only recent light level study examining the effects of tourniquet induced ischaemia-reperfusion injury that specifically looked at α -motor nerve terminals utilised YFP and mSOD1/YFP transgenic mice (David *et al.* 2007). With increasing numbers of the negative effects of YFP and without similar experiments carried out in wild-type animals, it is difficult to determine any potential contribution that XFPs may have made to results.

4.4.2 The responses of α -motor nerve terminals in real time: Pathophysiological or the ‘observer effect’.

Since the generation of the YFP transgenic lines of mice, there have been numerous live/repeat imaging studies. This has included imaging of dendritic spines of pyramidal neurons over hours, days and months during development (Grutzendler *et al.* 2002) sensory deprivation (Trachtenberg *et al.* 2002) and during/following ischaemic injury (Zhang *et al.* 2005). There have also been studies into the process of axotomy (Kerschensteiner *et al.* 2005) and developmental synapse elimination at the NMJ (Bishop *et al.* 2004), again over a time frame of minutes to months. There is however a certain degree of uncertainty in the results presented in all these studies due to the observer principal: are the observed changes truly physiological or are they the result of the act of looking (Lichtman and Fraser 2001)? This observer principal is particularly important in live/repeat imaging studies due to the inherent risk of inducing phototoxicity. For fluorophores, such as YFP, to fluoresce they must first absorb light energy (photons) to cause electronic excitation. In returning to their natural/ground

electronic state, fluorophores spontaneously emit photons but at a lower energy level (or longer wavelength) than which they originally absorbed and this is detected as the fluorescent signal. This ‘fluorescence’ mechanism however does not always occur: alternatively, excited fluorophores may return to ground state by donating their energy to other molecules, such as O₂, in a process known as ‘quenching’. This results in radicalisation (e.g., ROS formation) that causes damage to macromolecules, enzymes and cell membranes that may alter or cause dynamic responses and possibly lead to cell death, a process that is collectively referred to as phototoxicity. It is therefore imperative to reduce the light exposure as much as possible but some degree of phototoxicity is always going to occur in response to live/repeat imaging (Lichtman and Sanes 2003). In the preliminary results presented here and despite attempts to reduce light exposure as much as possible, it seems very unlikely that the response of α -motor nerve terminals to hypoxia was truly pathophysiological. This is primarily because α -motor nerve terminals that were not repeatedly imaged and away from the field of light did not have any observable pathology in response to hypoxia. As discussed above, the mice used in this study already exhibit a basal level of pathology and potentially express YFP at very high levels that would increase the risk of inducing phototoxicity. In line with previous reports of YFP induced toxicity and sensitising cell lines to other stimuli (see above), the most likely explanation for the results presented here is that hypoxia and the live imaging protocol acted as co-stimuli to cause the observed pathology in α -motor nerve terminals. The results presented here also contrast with the results obtained in wild-type TA muscles from animals of a similar age subjected to the 2H-2R protocol described in Chapters 2 and 3, where ~58% of endplates had no opposing NF/SV₂ immunoreactivity. The lack of pathology in terminals that had not been repeatedly imaged described here suggests that the level of hypoxia induced during the live imaging protocol was perhaps not as severe as that induced by the 2H-2R model system. This adds further evidence to indicate that the α -motor nerve terminals being repeatedly imaged were not responding to hypoxia alone. Phototoxicity is also probably responsible for the observation of a sprout-like event in control preparations. Numerous studies have addressed the issue of α -motor nerve terminal stability over time and have reached the conclusion that they are remarkably stable once synapse elimination is complete and until at least 18 months of age (Lichtman *et al.* 1987; Balice-Gordon and Lichtman 1990; Sanes and Lichtman 1999; Lichtman and Sanes 2003). Even the study that described the highest frequency of changes in gross morphology suggests that in mouse soleus each α -motor nerve terminal may only undergo the loss or addition of one terminal branch every 10 days (Wernig *et al.* 1984). It therefore seems unlikely that α -motor nerve terminal sprout event observed within 15 min in control conditions reflects physiological dynamism/plasticity. The live imaging system presented here is very similar to a very recently published protocol by Kerschensteiner *et al.* (2008) but these authors utilised TS muscles (rather than TA muscles: see Chapter 3) from a range of XFP expressing transgenic mice. They show that α -motor nerve terminals can be repeatedly imaged over several hours to examine, for example, developmental synapse elimination. They state that superfusion rates of 1 ml per min (total chamber volume of >3.5 ml) with Neurobasal A medium (preferred) or normal saline solutions

are suitable for these types of experiments. They do however imply that phototoxicity is a significant risk and utilise a computer controlled shutter system and neutral density filters (number unspecified), further advocating the use of intensified or electron multiplying CCD cameras to reduce overall light exposure. It is possible that by using improved microscope technology (i.e., computerised shutter controls and more sensitive camera systems) that overall light exposure was reduced enough to prevent significant amounts of phototoxicity than appears to have occurred in the present study. Nevertheless, this protocol developed by Kerschensteiner *et al.* (2008) has yet to be used to live/repeatedly image α -motor nerve terminal responses to pathological stimuli where perhaps it is more likely that the negative effects of live imaging/phototoxicity will be observed. Overall, the results presented here indicate that a certain amount of caution is required in interpreting the results of live imaging studies from XPF transgenic animals, especially in transgenic lines that appear to express very high levels of fluorescent protein such as those described here.

4.4.3 Live imaging reveals very rapid and potentially dynamic responses may occur in α -motor nerve terminals following a pathological insult.

Despite the fact that the results presented here are most likely due to some degree of phototoxicity and hypoxia, they do demonstrate that α -motor nerve terminals may respond very rapidly to pathological stimuli. Changes in morphology were observed to occur within 15min after the onset of live imaging and hypoxia but there are some difficulties in attempting to define what these events mean in regards to morphology. For example, do the sprout-like events observed in controls (Fig 4.4) and during hypoxia (Fig 4.5) represent sprouts like those known to occur following partial denervation and conduction block that are capable of innervating vacant endplates (Duchen and Strich 1968; Duchen 1970; Lichtman *et al.* 1987; de Paiva *et al.* 1999; Tam and Gordon 2003; Rogozhin *et al.* 2008), and reported to grow at 1.4 μ m per hr (Huang and Keynes 1983)? Alternatively, do these sprout-like events represent deformation/blebbing of presynaptic membrane? The swelling/fragmentation event marked by the asterisk in Fig 4.5: does this represent lysis of the α -motor nerve membrane and release of YFP, where the isolated 'fragments' related to trapped YFP deposits in and around the NMJ? Alternatively, are these 'fragments' membrane bound? These fragments may not be dissimilar to the axosomes described by Bishop *et al.* (2004) in their repeat/live imaging study, where axosomes were released from retreating α -motor nerve terminals during developmental synapse elimination and were later engulfed by Schwann cells. At the end of the hypoxia experiments, α -motor nerve terminals appeared with much reduced levels of YFP and composed of YFP puncta. Was this caused by a generalised release of YFP due to lysis/disruption of the presynaptic membrane? If so, where does the YFP go, especially considering that α -motor nerve terminals are surrounded by terminal Schwann cells, basal lamina and muscle fibres? Alternatively, was YFP and the general gross morphology of the α -motor nerve terminals still present but the YFP was no longer able to fluoresce, perhaps due to proteolytic breakdown? To try and address some of these possibilities, I attempted to image the

response of α -motor nerve terminal to LTX that is known to specifically cause large pore formation in presynaptic membranes, rapid swelling, lysis and necrosis of α -motor nerve terminals (Clark *et al.* 1970; Clark *et al.* 1972; O'Hanlon *et al.* 2003; Ushkaryov *et al.* 2004). Despite numerous attempts, experiments with LTX were generally unsuccessful due to the difficulty in keeping α -motor nerve terminals in the plane of view because of muscle fibre contractures. In the one successful experiment, YFP appeared to be lost in an asynchronous manner with some evidence of swelling but there was no fragmentation or obvious YFP release events. Due to some similarities between the responses of α -motor nerve terminals to LTX and hypoxia, this suggests that perhaps the results found in the hypoxic experiments were overall, necrotic. Nevertheless, the results observed during the LTX experiment need to be treated with extreme caution due to the lack of repeats, lack of superperfusion and the likelihood that there was also a significant contribution from phototoxicity, especially as they were imaged every 5min. Overall these results suggest that α -motor nerve terminals may respond in a highly dynamic manner to pathological stimuli but what exactly the observed events mean, as well as the cellular/molecular mechanism underlying them, are still unclear.

4.4.4 Summary

The probable contribution of phototoxicity and the general difficulty in interpreting results in the live/repeat imaging experiments on top of a basal level of pathology in the YFP transgenic animals used in this pilot study was the reason why this line of investigation was abandoned. With improved technology (e.g., computer controlled shutters systems and more sensitive light collection) it may be possible that future studies can be carried out to provide important information on the dynamic and spatiotemporal responses of α -motor nerve terminals by repeated/live imaging. There will however remain the constant problem of fully distinguishing true physiological or patho-physiological responses from that induced, even in part, by phototoxicity. It therefore seems prudent to suggest, especially based on the preliminary results presented here, that any novel findings from YFP transgenic mice and live/repeat imaging studies should be treated with some degree of caution and wherever possible, backed-up with studies in wild-type animals. This study also highlights potential problems with using YFP/XFP transgenic mice to replace traditional immunohistochemical methods in fixed tissues to observe neuron morphology as YFP does not appear to be totally innocuous and expression levels may vary considerably between mice and between colonies. The result presented here suggested that careful colony management of these mice maybe needed to prevent overly high expression levels or that other transgenic lines need to be developed where XFP expression is known/controlled, with known gene insertion sites, to help prevent future potential for unknown and unwanted effects of XFP expression.

Chapter 5

Expression of neuroglobin at the mouse neuromuscular junction: A pilot study

5.1 Introduction

Neuroglobin (NGB) is a recently identified member of the mammalian globin family, which, like its distant relative's haemoglobin, myoglobin and cytoglobin, can bind, store, and release oxygen (Burmester *et al.* 2000; Dewilde *et al.* 2001). NGB is highly expressed in mammalian neurons (Burmester *et al.* 2000; Mammen *et al.* 2002; Wystub *et al.* 2003; Laufs *et al.* 2004) and has O₂ binding/release kinetics that suggests that it will only release O₂ when demand is high (Dewilde *et al.* 2001). This indicates that NGB may play a specific role in maintaining neuronal O₂ homeostasis by acting as a buffer against transient hypoxic/ischaemic insults. NGB over-expression can confer protection to cultured cells and neurons *in vivo* against hypoxic/ischaemic insults and oxidative stress (Sun *et al.* 2001; Sun *et al.* 2003; Fordel *et al.* 2006; Fordel *et al.* 2007a; Fordel *et al.* 2007b; Li *et al.* 2007; Jin *et al.* 2008; Li *et al.* 2008), and during prolonged hypoxic insults, NGB mRNA/protein has been shown to be significantly up-regulated and to remain elevated for over 50hr after an hypoxic insult (Fordel *et al.* 2007a). This would suggest that changes in NGB expression levels may also be an important long term adaptive response to reduced oxygen levels and there is some, although conflicting, correlation between NGB expression levels *in vivo* with neuronal populations known to be vulnerable to oxidative stress and other neurodegenerative stimuli (Burmester *et al.* 2000; Moens and Dewilde 2000; Wystub *et al.* 2003; Sun *et al.* 2005). NGB expression levels have recently been demonstrated to compartmentalise into cell bodies and dendrites, with only very low expression in axons (Hundahl *et al.* 2008). Unfortunately, axonal nerve terminals were not studied but this indicates that NGB is localised to regions of neurons that are known to have the highest metabolic demands (Wong-Riley 1989; Attwell and Laughlin 2001; Hollenbeck and Saxton 2005) to suggest that it may also be significantly expressed at nerve terminals.

α -motor nerve terminals of the neuromuscular junction (NMJ) are likely to have equally high-energy demands as nerve terminals in the CNS but unlike the CNS, may be regularly subjected to hypoxic/ischaemic insults. Decreases in blood supply to peripheral limbs that occur as part of the body's thermoregulatory processes to maintain core temperatures, delays in capillary bed expansion during rapid rest-to-work transitions, intermittent blood supply at rest and lateral forces produced during strong muscle contractions may all cause temporary hypoxia/ischaemia (Shibata *et al.* 2005; Kernell 2006; Clanton 2007). Despite being regularly subjected to these types of insults, almost nothing is known about the metabolic and oxidative buffering abilities of α -motor nerve terminals. As NGB is known to be expressed in numerous locations in the spinal cord including the cell bodies of α -motor neurons (Stefan Reuss of Johannes Gutenberg University, personal communication; Mammen *et al.* 2002; Reuss *et al.* 2002; Wystub *et al.* 2003; Fordel *et al.* 2004; Hundahl *et al.* 2008), I report the preliminary findings from a study examining the presence of NGB at the mouse NMJ.

5.2 Methods

5.2.1 Animals and skeletal muscle dissection

Triangularis sterni (TS) muscles from 8-12wk female C57Bl/6 mice were dissected in silicone-lined petri dish and maintained in HEPES buffered Krebs' solution as previously described in Chapter 3 (section 3.2.1).

5.2.2 Immunohistochemistry

Following dissection, tissues were quickly fixed by immersion in freshly made PLP (4% formaldehyde (Electron Microscopy Sciences, PA 19440, USA), 1.8% lysine, 0.2% sodium-m-periodate; McLean and Nakane 1974; Reuss *et al.* 2002; Schmidt *et al.* 2003; Wystub *et al.* 2003) for 30min at room temperature. Alternatively, tissues were immersion fixed in absolute MeOH at -20 °C for 15min. Fixed tissues were rinsed (3x10min) in 1% TritonX-100 in PBS, underwent final dissection and were incubated in blocking solution (0.2% IgG free & protease free bovine serum albumin (Jackson ImmunoResearch, via Stratech, UK); 0.3% TritonX-100, 0.1% sodium azide in PBS, pH 7.2) for a 1hr(min). On occasion blocking solution was supplemented with 0.3% donkey serum (DAKO) to help reduce non-specific binding of antibodies, particularly to the muscle connective tissue. TS muscles were incubated in polyclonal primary rabbit anti-neuroglobin (NGB: 1:100) or rabbit anti-s100 (1:250: Dako) antibodies in blocking solution overnight at 4°C. The polyclonal anti-NGB antibody was a generous gift from Stefan Reuss (Johannes Gutenberg University, Germany) and was raised against a conserved 55-70 amino acid sequence of the human and mouse NGB recombinant protein (H₂N-CLSSPEFLDHIRKVML-CONH₂). The specificity of the anti-NGB antibody has been previously confirmed (Reuss *et al.* 2002; Schmidt *et al.* 2003; Wystub *et al.* 2003). Following a rinse in blocking solution for 3x10min, preparations were incubated in Cyanine2 (Cy2)-conjugated donkey anti-rabbit IgG (1:500 in blocking solution: Jackson ImmunoResearch, via Stratech, UK) for 1h at room temperature. Post-synaptic acetylcholine receptors were then labelled by incubation in TRITC-conjugated BTX for 30mins (5µg/ml in PBS: Invitrogen). After a minimum of 4x10min washes in PBS, muscles were mounted onto slides using 4% n-propylgallate in glycerol and stored in the dark at 4°C.

5.2.3 Image Capture

All preparations were examined on an upright Zeiss microscope equipped with epifluorescence FITC/TRITC filter sets and fluorescent Leitz water-immersion objectives (x25 NA 0.6, x50 NA 0.8: see Chapter 2 for further details). *N*= number of animals, *n*= number of muscles (up to two TS muscles per animal)

5.3 Results

Immunoreactivity of NGB at the neuromuscular junctions (NMJs) of TS muscles fixed in PLP and MeOH is shown in Fig 5.1(A-C). Despite selecting a very thin, planar muscle to enhance antibody penetration, changing the type of fixative and trying to reduce non-specific binding of antibodies with the addition of donkey serum (see methods), NGB immunoreactivity was, in general poor and of very low intensity. In regions of muscle where overlying connective tissue had been successfully removed, non-specific binding of antibodies and autofluorescence was low, NGB immunoreactivity could however be observed to localise at NMJs. In PLP fixed tissue ($N=4$, $n=4$), NGB immunoreactivity can be seen to almost directly match the characteristic 'pretzel' morphology of the post-synaptic endplate (visualised with BTX). Careful comparison of BTX with NGB staining shows very small but discrete interlinking regions of NGB immunoreactivity that are not opposed by BTX (Fig 5.1A: arrowhead). This is a typical staining characteristic of an antigen in a presynaptic location, corresponding to interlinking synaptic boutons (Baxter *et al.* 2005). Further evidence of a presynaptic location is evidenced by what appears to be pre-terminal staining of axons and/or Schwann cells (Fig 5.1A (arrow)). It is unclear however, if this presynaptic staining is due to the presence of NGB in motor nerve axons, and/or terminals, and/or Schwann cells. Fig 5.1D shows the typical morphology of Schwann cells (visualised by S100 immunoreactivity, a Schwann cell specific protein) at the NMJ of mouse TS muscle. Schwann cells are distinguishable from motor nerve axons and terminals (see main thesis for details of nerve terminal morphology and staining characteristics) by their cell bodies and by regular breaks in staining at the nodes of Ranvier. Similar staining characteristics can also be found at NMJs stained for NGB, with cell body-like structures (Fig 5.1B) and nodal-like breaks (Fig 5.1A). These nodal-like breaks would suggest that NGB is perhaps not located in motor nerve axons. Diffuse staining of NGB also appears around the endplate zone, suggestive of a postsynaptic location but not muscle fibres as a whole. Muscle fibres in general showed no fluorescence signal above what could be attributed to autofluorescence. In TS muscle fixed with MeOH ($N=1$, $n=1$: Fig5.1C), NGB immunoreactivity was much more confined and almost the complete reverse of the staining found in PLP fixed tissue with NGB immunoreactivity appearing to edge the NMJ boundary as defined by BTX staining. Again, it is unclear what location at the NMJ this staining may represent as without careful co-localisation studies with other known antigens, this could conceivably be presynaptic, postsynaptic or even perhaps staining of the basal lamina adjacent to the synaptic cleft. Why different fixatives should result in such vastly different NGB staining patterns is also unclear. Overall, these preliminary results suggest that NGB may be located at the NMJ and is most suggestive of being located in Schwann cells but, NGB could also be potentially located in motor nerve terminals and/or postsynaptic endplates/muscle fibres. Due to poor immunoreactivity, however these results are by no means conclusive and highlight the need for further study.

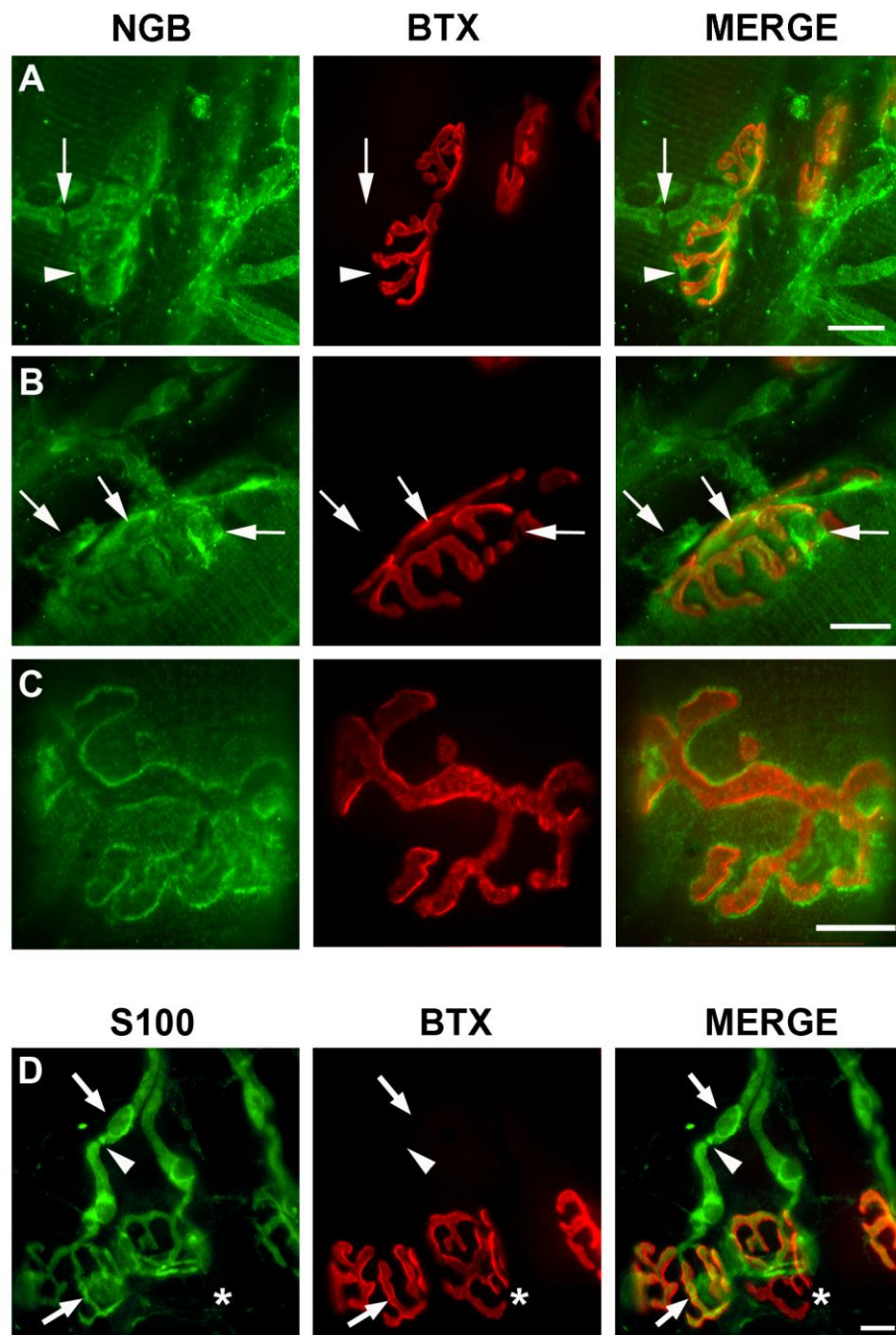


Fig 5.1 NGB immunoreactivity at the mouse NMJ. Double labelled mouse NMJs from TS muscles showing NGB (**A-C**) and S100 immunoreactivity (**D**: a Schwann cell specific protein) visualised with Cy2 secondary antibodies (GREEN). Postsynaptic endplates were labelled with TRITC conjugated BTX (RED) with tissues fixed in PLP shown in (A, B, & D) and tissues fixed in MeOH in (C). NGB can be observed to localise at the NMJ, with characteristic pretzel-like morphology that opposes BTX staining (most evident in B). NGB immunoreactivity can be seen to be interlinking with small discreet regions of NGB immunoreactivity not opposed by BTX that is suggestive of a presynaptic location (arrowhead in A). Presynaptic location is also indicated by the axonal-like projection in the arborisation of the NMJ (arrow in A). Low levels of diffuse staining can be seen to surround the endplate zone in (B). Compared with PLP fixed TS muscle (A&B), NGB immunoreactivity in TS muscle fixed in MeOH (C) is almost completely reversed. NGB is localised to the very edge of the NMJ, as defined by BTX staining. It is unclear what location at the NMJ this staining may represent, as this could conceivably be presynaptic, postsynaptic or even perhaps staining of the basal lamina adjacent to the synaptic cleft. (D) shows the typical morphology of Schwann cells from the mouse TS muscle. Several Schwann cells can be seen to overlie and closely correspond to the characteristic 'pretzel' morphology of the postsynaptic endplate. This is very similar to presynaptic staining of α -motor nerve terminals but Schwann cells can be distinguished from α -motor nerve axons and terminals by the presence of their cell bodies (arrows) and by the regular breaks in staining at the nodes of Ranvier (arrowhead). When the S100 Schwann cell staining is compared to that of NGB, there are some similar features. Numerous small swellings on the NMJ arborisation and incoming nerve look like the cell bodies of Schwann cell (arrows in B) and breaks in NGB immunoreactivity appear like nodes of Ranvier (arrow in A). Curiously in (D), approximately half of one endplate, with no apparent pathology, has no opposing S100 immunoreactivity (asterisk). This suggests that either the terminal Schwann cell is absent or is not S100 positive. The reason for this is entirely unknown. Scale bar 10 μ m.

5.4 Discussion

Almost nothing is known about how α -motor nerve terminals buffer against transient deficits in O₂ supply despite the fact that they may become hypoxic/ischaemic as a result of intermittent blood supply at rest, rapid rest-to-work transitions and impaired blood flow during muscle contraction (Shibata *et al.* 2005; Kernell 2006; Clanton 2007). Neuroglobin (NGB) is novel protein that has O₂ binding/release kinetics that is suggestive of a role in buffering against hypoxic insults (Dewilde *et al.* 2001). NGB is known to be expressed in the cytoplasm of rodent CNS neurons where it was first identified (Burmester *et al.* 2000) and NGB mRNA and/or protein has also been reported to be expressed in the peripheral autonomic nervous system, liver, heart and in the cell bodies of α -motor neurons in the spinal cord (Stefan Reuss, personal communication; Mammen *et al.* 2002; Reuss *et al.* 2002; Wystub *et al.* 2003; Fordel *et al.* 2004; Hundahl *et al.* 2008). The data presented here is the first study that examines the expression of NGB at the mouse NMJ and while this study was inconclusive in regards to the expression of NGB in α -motor nerve terminals, the results presented here provide some preliminary evidence of NGB expression at the NMJ.

5.4.1 Is NGB expressed at the NMJ?

In all immunohistochemical studies, and especially those where there is no prior knowledge of antigen location, it is essential that the specificity of the antibodies used are correctly determined to ensure that observed labelling patterns represent the true localisation of the antigen in question rather than being false-positive (or even false-negative) as a result of poor affinity, specificity or cross-reactivity of antibodies. In the preliminary study described here, control studies were not carried out however previous studies within the same laboratory, with the same muscle and same secondary antibodies have shown no immunoreactivity when primary antibodies are omitted (Vega-Riveroll *et al.* 2005) demonstrating that the secondary antibody does not bind unspecifically. There is also evidence to indicate the anti-NGB antibody is specific: The antibody was raised against a unique NGB amino acid sequence and detects NGB located in various regions in the mouse brain (e.g., cerebral cortex, cerebellum and purkinje cells) that was comparable to the localisation of NGB using mRNA in situ-hybridisation (Reuss *et al.* 2002; Wystub *et al.* 2003) and similar to other staining patterns using anti-NGB antibodies raised against different epitopes (e.g., Hundahl *et al.* 2008). Preabsorption of the primary anti-NGB antibody with its recombinant protein also blocked NGB staining in native mouse tissue samples. This, alongside western blotting where a single band was observed at the expected molecular weight (~17KDa) and ELISA¹¹ analysis (Wystub *et al.* 2003; Schmidt *et al.* 2003; Laufs *et al.* 2004) collectively provide some evidence for the specificity of the primary antibody used here but it is by no means conclusive. This is a general difficulty in all studies that rely on specific antibody binding, particularly in native tissue samples, in confirming specificity (e.g., Lorincz and Nusser

¹¹ ELISA: enzyme-linked immunosorbant assay

2008; Saper 2008). Antibodies can only recognise a limited number of amino acids on any given protein antigen and binding requires not only the correct sequence (which may or may not be linear) but also the correct 3D spatial configuration (Hayat 2002). Finding unique sequences is therefore difficult and despite the fact that an antibody may be raised against what appears to be a unique amino acid sequence, it may also react with other proteins that also have unexpected epitopes due to their spatial configuration. This cross reactivity is most likely to occur in related family proteins/isoforms (Lorincz and Nusser 2008) but can occur in any protein including those proteins yet to be identified. This fundamental problem with immunohistochemistry has led to numerous debates and calls for best practice to help ensure specificity, although this may never be guaranteed. Several authors have suggested that to confirm specificity of antibody labelling, immunohistochemical studies should be carried out in genetic “knock-out” animals but this is not without its own caveats that includes the viability of knock-out animals and changes to other proteins that result in false-positive immunoreactivity as a result of unexpected effects of gene knock-outs (e.g., Anderson and Nedergaard 2006; Lorincz and Nusser 2008; Saper 2008). Alternatively, a range of different antibodies can be used that are raised against different epitopes of the same antigen, and where the same staining patterns are found with different antibodies it can be assumed that antibody binding is indeed largely specific (as it is unlikely that different antibodies will all bind to produce the same staining pattern if they are not specific: Lorincz and Nusser 2008; Saper 2008). This method again however is also subject to its own caveats, as the cause(s) of different staining patterns can be difficult to interpret and may not necessarily mean antibodies bind unspecifically (see below). Many of the control procedures recommended for determining antibody specificity have already been carried out by others on the anti-NGB antibody used in this study (see above) however, it is important that additional controls are also carried out and specifically use neuromuscular tissues in future studies. This should undoubtedly include omission of the primary anti-NGB antibodies to conform that the secondary antibody is not binding unspecifically and preabsorption of the primary antibody to its recombinant protein antigen (although the value of this as a control procedure is highly questionable if the antibody is monoclonal or has been affinity purified (Saper 2008) as was done with the antibody used here). Further analysis should also confirm that the anti-NGB does not cross-react with its related family members (haemoglobin, myoglobin and cytoglobin) because as far as I am aware, this has yet to be definitively and formally addressed (again, the true value of this as a control is questionable as such analysis will most probably rely on western blotting techniques (or similar) which uses unfixed and solubilised/linear (and probably recombinant) protein extracts and therefore there is no guarantee of comparable results on the cross-reactivity of an antibody when examining results from native, fixed tissues with that of western blot (or similar) analysis (Lorincz and Nusser 2008; Saper 2008)). In addition to this, other antibodies raised against different epitopes of NGB should be used and staining patterns compared in positive control tissues and at the NMJ. In the future, it may also be possible to test anti-NGB antibodies in knock-out tissues should these animals be viable. With the addition of these controls, it will help to determine if the NGB staining observed in the current study is specific at the mouse NMJ but until these studies are completed and despite the fact this antibody has been

previously published as specific, the observation of NGB immunoreactivity at the mouse NMJ remains a preliminary result.

5.4.2 Where at the NMJ could NGB be expressed?

If it is assumed that the NGB antibody binding is largely specific as shown in previous publications (Wystab *et al.* 2003; Schmidt *et al.* 2003; Laufs *et al.* 2004), the observed immunofluorescent staining pattern of NGB at the NMJ was suggestive of a presynaptic location, especially in Schwann cells. There was also some suggestion that NGB may also be located postsynaptically at the NMJ but not generally in the muscle fibre as a whole, which is consistent with real time PCR and in-situ hybridisation studies in rodent skeletal muscle (Reuss *et al.* 2002; Fordel *et al.* 2004). These preliminary findings however cannot rule the possibility that NGB may also be expressed in α -motor nerve axons and terminals. In the mammalian brain, NGB only represents 0.01% (μ M range) of the total protein content (Burmester *et al.* 2000; Dewilde *et al.* 2001; although others have suggested a much higher concentration (Reuss *et al.* 2002)) and NGB has also been shown to be compartmentalised into cell bodies and dendrites, with very low expression in axons (Hundahl *et al.* 2008)). It is possible that a generally low level of NGB expression will explain the poor immunoreactivity found here and if NGB is also compartmentalised, lower levels of NGB in α -motor neuron axons may have rendered NGB immunoreactivity undetectable against background and possible Schwann cell fluorescence. The strongest evidence for the location of NGB at the NMJ however was for presynaptic Schwann cells due to the numerous cell body-like structures and breaks in immunoreactivity indicative of nodes of Ranvier. This preliminary result is surprising as NGB is thought to be neuron specific due to neuron-restrictive silencer element-like motifs in the human and mouse NGB gene that may actively repress expression in non-neuronal cells (Laufs *et al.* 2004) and no detectable expression has been found in glial cells in the mouse CNS or in glial cell lines (Wystub *et al.* 2003; Laufs *et al.* 2004)). In their mRNA in-situ hybridisation analysis, Reuss *et al.* (2002) however did identify some structures that were possibly a sub-population of specialised glial cells that may contain NGB in specific regions of the CNS. It may therefore be possible that NGB is expressed in a highly select group of glial cells and therefore raises the possibility that it may also be expressed in myelinating and terminal Schwann cells. From the preliminary results presented here, it may seem possible that NGB may be located in several compartments of the NMJ and therefore future studies should consider immunoprecipitation of diaminobenzidine (DAB) or similar techniques. This method involves significant amplification of immunoreactivity due to the increasing antibody binding ratio of biotinylated antibodies and of the peroxidase reaction which would help overcome the poor fluorescent immunoreactivity noted here (Hayat 2002). DAB is also electron dense and with further tissue processing, would allow the localisation of NGB to be resolved to the sub-cellular level with electron microscopy. This technique was attempted numerous times in the current study however, a range of technical difficulties (no immunoreactivity could be achieved probably due to sodium azide contamination) meant that this line of investigation was unsuccessful and was abandoned.

The reasons underlying the changes in NGB immunoreactivity achieved with different fixatives is unknown. PLP fixative, that has been used in several of the studies examining NGB expression in rodents (Reuss *et al.* 2002; Schmidt *et al.* 2003; Wystub *et al.* 2003), contains formaldehyde with the addition of lysine and periodate, and can stabilise tissue carbohydrate as well as amino acids by chemical cross-linking (McLean and Nakane 1974; Hayat 2002). MeOH is a relatively mild precipitant fixative that causes coagulation of proteins in the absence of chemical cross-linking (Hayat 2002). As already mentioned epitope recognition by an antibody is highly reliant on spatial configuration that can be easily altered by the process of fixation to bring about a change in antigen structure or physically prevent the antibody gaining access to the epitope that can be later reflected in observed staining patterns (e.g., Hayat 2002; Lorincz and Nusser 2008). Antibody-epitope recognition is also affected by a whole range of factors that not only includes type of fixative, but also duration, temperature, pH, detergents, blocking steps, ionic concentration and additional antigen/epitope retrieval steps of the entire immunohistochemical experimental process (Hayat 2002; Lorincz and Nusser 2008). Overall, this means that epitopes may become ‘masked’ or ‘unmasked’ by different immunohistological processing techniques, particularly if they are located in different cellular compartments and/or spatially linked to different proteins/structures (that may in turn respond differently to the experimental procedure to contribute to masking/unmasking of epitopes) to potentially produce very different staining patterns (reviewed, with numerous examples, by Hayat 2002; Lorincz and Nusser 2008). When different staining patterns are achieved it can be difficult to determine the false-positive (or –negative) results from the true localisation, or indeed if both staining patterns are proved correct, the reason underlying the differences but if carefully analysed, such results may provide important clues about different sub-cellular locations of an antigen (Lorincz and Nusser 2008). For example the differences in observed staining patterns in MeOH and PLP fixed tissues could have been caused by alternate epitope masking and unmasking due to the different fixatives having different effects on the antigen. Different NGB staining patterns could also be possibly attributed to NGB being located in different cellular compartments that may have had different pH or Ca^{2+} concentrations for example and/or because NGB is located with different complexes (e.g., different accessory proteins). All of these possible factors in turn could have responded differently to the change in fixative (and to the immunohistochemical protocol as a whole) to cause varying degrees of epitope masking/unmasking and ultimately, led to different staining patterns. A further level of complexity is added when it is considered that the anti-NGB antibody used here is polyclonal. Alternatively, one or both staining patterns observed with different fixatives could have been false positive due to non-specific binding or cross reactivity of the antibodies. This highlights the need for further studies regarding the expression of NGB at the NMJ and that this needs to be done with careful consideration and methodical investigation of the various factors that can influence antibody-epitope binding (e.g., fixation, blocking steps and antigen retrieval techniques). This process would be helped by using antibodies raised against different epitopes of NGB, and with careful comparison and interpretation of staining patterns under various immunohistochemical

experimental processes, to help determine if NGB is located at the NMJ. The potential caveats of immunohistochemical protocols also highlight the need to use a broad range of other investigative techniques (e.g., pharmacology, genetic and functional) to look at NGB expression at the NMJ. Only a combination of results from a range of techniques, as each has its own caveats, will be able to prove or disprove if NGB is located at the NMJ. The preliminary results presented do however provide a starting point for basing further study.

5.4.3 If NGB is expressed at the NMJ, what is its role?

As a member of the globin family, NGB has the capacity to bind, store and release O₂ helping to maintain supplies of oxygen for oxidative phosphorylation of ATP. NGB has also been shown to be a reactive oxygen species scavenger (Fordel *et al.* 2006; Fordel *et al.* 2007b; Jin *et al.* 2008; Li *et al.* 2008), to have some additional sodium dismutase activity as well as being able to act as an NADH oxidase, facilitating continuing ATP production in oxygen limiting conditions (Dewilde *et al.* 2001; Trandafir *et al.* 2007; Giuffre *et al.* 2008). There is still some controversy over the relative physiological importance and hierarchy of NGBs metabolic properties but NGB has been shown to convey protection during oxidative injury both *in vivo* and *in vitro* (Sun *et al.* 2001; Sun *et al.* 2003; Fordel *et al.* 2006; Fordel *et al.* 2007a; Fordel *et al.* 2007b; Li *et al.* 2007; Jin *et al.* 2008; Li *et al.* 2008). NGB has also been shown to attenuate β -amyloid pathology in culture, a major pathological hallmark of Alzheimer's disease (Li *et al.* 2007). The preliminary evidence that indicates the presence of NGB at the NMJ suggests a potentially important role in oxygen and metabolic homeostasis, and a defence mechanism against transient decreases in O₂ levels. In particular, NGB may have a key role in O₂/metabolic buffering in Schwann cells, based on the preliminary fluorescent immunohistochemical staining characteristics described here. Schwann cells play a vital role in the development, maintenance, plasticity and pathological responses of α -motor nerve terminals by providing for example trophic support and cues (Balice-Gordon 1996; Son *et al.* 1996; Yin *et al.* 2004; De Winter *et al.* 2006). Little is known about their metabolic properties, their vulnerability to hypoxia/ischaemia-reperfusion injuries or their potential involvement in the pathogenesis of similar injuries in α -motor neurons (a situation that should be addressed in future studies) but NGB expression in Schwann cells perhaps suggests that they may have an improved ability to buffer against these types of insults. It is interesting to note that in invertebrates, neuronal myoglobin is highly expressed in glial cells with expression levels being particularly high in those of the mollusc living in hypoxic environments (Dewilde *et al.* 1996; Reuss *et al.* 2002). This suggests a potentially important role for NGB in glial cells in helping to maintain neuronal O₂/metabolic homeostasis. Further investigations of the potential role of NGB at the NMJ are needed and perhaps further insights may be gained from using the hypoxia-reperfusion model system developed here (described in Chapters 2 and 3) and utilising tissue from the recently generated NGB over-expressing mouse (Khan *et al.* 2007). Our understanding of the role of NGB at the NMJ however can only be realistically achieved once the expression of NGB is

confirmed at the NMJ and the exact cellular location is determined, both of which are still unclear from the results of present study.

5.4.4 Summary

In summary, NGB is a recently identified member of the oxygen-carrying family of globins and preliminary results suggest that it may be expressed at the mouse NMJ. The preliminary fluorescent immunohistochemical staining provided evidence to suggest that NGB may be located in Schwann cells but there was also some evidence that it could also be expressed postsynaptically and possible expression in presynaptic nerve terminals cannot be ruled out. The preliminary results presented here suggest there may be a possible role for NGB in the O₂ and metabolic buffering of NMJs against transient hypoxic/ischaemic insults. I do however highlight numerous difficulties in determining antibody specificity and interpreting immunohistochemical data in studies such as the pilot study described here. To help overcome some of these difficulties I further suggest that to confirm or disprove the presented preliminary findings of NGB expression at the mouse NMJ, future work should use a range of antibodies raised against different NGB epitopes, where each antibody is proven to bind specifically and subjected to a range of different immunohistochemical staining protocols. Following on from studies confirming antibody specificity, the exact location of NGB (Schwann cell, postsynaptic endplate, motor nerve axon and/or terminal) can be further investigated, however NGB expression at the NMJ can only be realistically confirmed with a range of techniques including functional, pharmacological and genetic due to the inherent pitfalls of immunohistochemistry.

Chapter 6

General Discussion

6. General Discussion

There is increasing evidence to support the notion that neurons can be considered compartmentalised with cell bodies, axons, and nerve terminals having many discrete structural, functional, and metabolic characteristics. Neurons also appear to be compartmentalised in terms of the vulnerability to pathological stimuli with nerve terminals in particular often being the principal site of functional and structural changes in many traumatic, toxic and neurodegenerative disease conditions (e.g. Raff *et al.* 2002; Wishart *et al.* 2006; Conforti *et al.* 2007a: see Table 1). The underlying reasons for this are unknown, but changes to and loss of nerve terminals undoubtedly cause aberrant functional changes due to loss of synaptic connectivity and trigger many of the punitive symptoms that characterise neuropathological conditions. One possible explanation for the vulnerability of nerve terminals is their high bioenergetic demands (Wong-Riley 1989; Attwell and Laughlin 2001; Miller and Sheetz 2004; Hollenbeck and Saxton 2005). The mitochondria contained within nerve terminals however are more susceptible to metabolic inhibition, have accumulated oxidative damage, reduced Ca^{2+} buffering capacity and are more susceptible to rupture, that particularly affect the longest of neurons (e.g., α -motor neurons of the lower limbs: John 1996; Davey *et al.* 1998; Brown *et al.* 2006; Naga *et al.* 2007). This would clearly imply that nerve terminals would be highly vulnerable to pathological stimuli aimed directly at mitochondria and inhibition of aerobic respiration, such as hypoxia/ischaemia. In the CNS, the role of nerve terminals in mediating the devastating and often fatal consequences of ischaemic injuries (where the hypoxic element is the key pathological stimulus) are well known, but our understanding of similar injuries in the PNS are at best, poor. This is of particular clinical importance as up to 1 in 11,000 of the UK population suffer paralysis and long term neurological complications following ischaemia-reperfusion injuries caused by the application of surgical and non-surgical tourniquets (e.g., Bernard Roth 1931; Eckhoff 1931; McElvenny 1945; Rudge 1974; Saunders *et al.* 1979; Rorabeck 1980; McGraw and McEwen 1987; Kalla *et al.* 2003; Kam 2007; Murphy and O'Connor 2007). To help develop preventative or therapeutic strategies to address this problem it is vital to determine the underlying causes and the principal pathological target of such stimuli. There have been several suggestions that the nerve terminals of α -motor neurons maybe the principal site of injury following hypoxia/ischaemia but there has not yet been a conclusive study that has addressed this possibility.

6.1 What are the key findings and long-term implications of the present study?

By using a novel experimental model system, I have demonstrated the rapid and selective vulnerability of *ex vivo* mouse α -motor nerve terminals to hypoxia-reperfusion injury. Following 2hr hypoxia-2hr reperfusion (2H-2R), I show significant loss of presynaptic neurofilament and synaptic vesicle protein (NF/SV₂) immunoreactivity, disruption of the microtubule cytoskeleton and functional

loss of α -motor nerve terminals and pre-terminal axons. That was collectively indicative of an ongoing process of α -motor nerve terminal disassembly, while postsynaptic endplates and muscle fibres appeared unaffected by 2H-2R. The severity of these pathological changes was age and muscle type dependent: in 8-12wk old mice, ~83% of postsynaptic endplates appeared vacant (with no opposing presynaptic NF/SV₂ immunoreactivity) in the predominantly fast-twitch lumbricals but in the predominantly slow-twitch TA and TS muscles, only 58% and 28% of endplates were vacant respectively. In juvenile mice (5-6wks) the response of TA and TS muscles was markedly increased with >97% of endplates appearing vacant while lumbrical muscles appeared largely unaffected by age. I also show that neuroprotective *Wld^s* mutation does not mitigate the pathology caused by 2H-2R. Overall, I have demonstrated that significant pathology at α -motor nerve terminals occurs as a result of changing O₂ levels where the hypoxic phase was carried out at levels below those reported for *in vivo* normoxia (0.5%- 4%: Gorczynski and Duling 1978; Klitzman *et al.* 1983; Honig and Gayeski 1993; Shibata *et al.* 2001; Eu *et al.* 2003; Matsumoto *et al.* 2005) and similar to levels reported to occur during tourniquet induced ischaemia (0.25%: Gustafsson *et al.* 1999; Matsumoto *et al.* 2005). α -motor nerve terminal pathology also occurred as a result of a clinically relevant stimulus of 2hr hypoxia, the same time frame that is generally agreed to be the upper limit for tourniquet application (McElvenny 1945; Kessler 1966; Rorabeck 1980; Pedowitz *et al.* 1992; Kalla *et al.* 2003; Younger *et al.* 2005; Malanjam and Fischer 2006; AORN 2007; Kam 2007). This study therefore contrasts with previous studies of *ex vivo* α -motor nerve terminal responses to hypoxia that have only described reversible functional changes at highly variable (and possibly non-hypoxic) or undefined O₂ levels (Krnjevic and Miledi 1959; Hubbard and Loyning 1966; Anwyl and Ling 1983; Nishimura 1986; Bazzzy 1994; Zhu *et al.* 2006). It also contrasts with previous *in vivo* studies of ischaemia-reperfusion injuries where α -motor nerve terminal pathology could not be conclusively distinguished from tourniquet induced ischaemia-reperfusion injury, tourniquet induced mechanical trauma or that of postsynaptic/muscle fibre damage (Makitie and Teravainen 1977a; Diwan and Milburn 1986; Tombol *et al.* 2002; Eastlack *et al.* 2004; David *et al.* 2007). Furthermore, these *in vivo* studies could not distinguish between the relative importance of hypoxia, hypoglycaemia, acidosis, gross ionic changes, gross inflammation and toxic metabolite build up that are all known to occur in response to ischaemia-reperfusion injuries (see Chapter 1, sections 1. 6.1 and 1.7.2). Some of the neurological complications following the use of tourniquets may occur because they are incorrectly applied and/or over-pressured, triggering mechanical trauma to underlying nerves (Eckhoff 1931; McElvenny 1945; Fowler *et al.* 1972; Ochoa *et al.* 1972; Rydevik and Nordborg 1980; Ohara *et al.* 1996; Kam *et al.* 2001; Kalla *et al.* 2003). From the results presented here however, functional and structural pathology at α -motor nerve terminals distal to tourniquets and as a result of ischaemia-reperfusion injury may also significantly contribute to, or perhaps in some cases cause, the neurological complications following the application of tourniquets.

In further support of the finding that α -motor nerve terminal pathology distal to tourniquets may be contributing to injury, it seems likely that the extent of pathology observed in the current study would underestimate the full extent of pathology over a similar same time frame *in vivo*. It has been previously noted that in general, *ex vivo* models of CNS hypoxia/ischaemia-reperfusion injuries are typically much slower to respond compared with *in vivo* model systems (Lipton 1999; Kovalenko *et al.* 2006). In the current study, experiments were carried out in the presence of glucose at room temperature but glucose is known to delay the onset of pathology by supporting anaerobic respiration (Allen *et al.* 2005) and hypothermia has also been shown to be a very effective neuroprotectant in ischaemia-reperfusion injuries in peripheral nerves (Kihara *et al.* 1996; Mitsui *et al.* 1999b; Mitsui *et al.* 1999c; Kawamura *et al.* 2005; Kawamura *et al.* 2006). It is also well accepted that the full extent of pathology following *in vivo* ischaemic injuries takes days to weeks to develop in the both the CNS (e.g., Nakano *et al.* 1990; Lipton 1999; Kovalenko *et al.* 2006) and in the periphery (Fowler *et al.* 1972; Makitie and Teravainen 1977b; a; Zollman *et al.* 1991; Pedowitz *et al.* 1992; Nukada and McMorran 1994; Carden and Granger 2000; Tombol *et al.* 2002; Iida *et al.* 2003; Wang *et al.* 2008). Could the evidence of an on-going process of α -motor nerve terminal disassembly described here have developed into gross structural loss of α -motor nerve terminals: given a longer reperfusion period; at more physiologically relevant temperatures; or as a result of an ischaemic rather than an hypoxic stimulus? Could this be followed by retrograde degeneration and even loss of α -motor neurons? Studies that have examined the effects of the direct disruption of synaptic vesicle release (Pastor *et al.* 1997; Berliocchi *et al.* 2005) and microtubules (Hsu and Lentz 1972; Wang *et al.* 2000; Wang *et al.* 2001), not unlike that described here (Chapter 2), have shown that these types of stimuli can not only cause α -motor nerve terminal loss but also dying back neuropathies. Furthermore, a large range of other pathological stimuli that cause similar changes in nerve terminals are also known to cause gross α -motor nerve loss and dying back neuropathies (see Chapter 1, section 1.6 and Table 1). The structural events described here are also known to occur during CNS hypoxia/ischaemia-reperfusion injuries where swelling, vacuolisation, reduced synaptic vesicle density, loss of pre-synaptic proteins critical for regulating synaptic vesicle turnover have been identified prior to other structural changes in axons or cell bodies (e.g., Williams and Grossman 1970; Rothman 1983; von Lubitz and Diemer 1983; Nishimura *et al.* 2000; Ishimaru *et al.* 2001; Bolay *et al.* 2002; Jourdain *et al.* 2002; Kovalenko *et al.* 2006). These selective structural and functional events at nerve terminals have been suggested to either signal back to the cell body to cause cell death or trigger retrograde cell death (Martone *et al.* 1999; Nishimura *et al.* 2000; Ishimaru *et al.* 2001; Bolay *et al.* 2002; Jourdain *et al.* 2002). From the results presented here, hypoxia-reperfusion injury certainly does appear to cause some retrograde pathology as NF/SV₂ staining of pre-terminal axons was often absent but present in the larger intramuscular axon bundles to indicate a distal to proximal progression. It is however unclear at present if pre-terminal axon pathology was caused by retrograde spreading of α -motor nerve terminal pathology or if axons were responding to 2H-2R *per se*. Considering that the average orthopaedic surgeon will apply ~200 tourniquets per annum (Middleton and Varian 1974) and that

>60% of patients will suffer long-term (>6months) neurological complications following the use of tourniquets (Saunders *et al.* 1979), the fate of α -motor nerve terminals, axons and possibly even cell bodies in response to hypoxic/ischaemia-reperfusion injuries are important questions to answer in future studies as this will influence if, and how long it may take to recover from injuries of this type. α -motor neurons have considerable regenerative abilities and are able to recover relatively rapidly even from complete denervation as long as the cell body, muscle fibres and Schwann cells remain viable. Over a period of weeks to months surviving proximal nerve stumps following axotomy are 'guided' by Schwann cells to re-innervate skeletal muscle (Brown *et al.* 1976; Gordon and Stein 1982; Gordon *et al.* 1993; Son and Thompson 1995; Son *et al.* 1996; Costanzo *et al.* 1999; Costanzo *et al.* 2000) but this process of *de novo* innervation can take many months to complete, linked to permanent changes in the dendritic tree (Sumner and Watson 1971; Brännström *et al.* 1992) and in some cases it can be very protracted and incomplete (Gordon and Stein 1982; Gordon *et al.* 1993; Fu and Gordon 1995; Rafuse and Gordon 1998). Recovery of skeletal muscle function can also be produced by a local mechanism of collateral re-innervation or 'sprouting' by neighbouring α -motor nerve terminals and pre-terminal axons (specifically, at the nodes of Ranvier) that send projections into vacant postsynaptic endplates (Tam and Gordon 2003). Sprouting is a common injury response following, for example, partial denervation (Gordon *et al.* 1993; Frey *et al.* 2000) or application of Botulinum A toxin (Duchen and Strich 1968; Duchen 1970; Pastor *et al.* 1997; de Paiva *et al.* 1999; Meunier *et al.* 2002) and can occur very rapidly with sprouts reported to grow at a rate of 1.4 μ m per hr (Huang and Keynes 1983). The resulting increase in motor unit size can compensate for up to an 80% loss of α -motor neurons (Gordon *et al.* 1993) but significant and permanent collateral innervation may cause 'stress' leading to an inability to maintain these excessively large motor units triggering delayed neuronal death (Kernell 2006). Signs of ultrastructural recovery of α -motor nerve terminals following tourniquet induced ischaemia-reperfusion injury have been described by Tombol *et al.* (2002) after 1wk but recovery was not complete by the end of the 4wk experiment, with between 26% and 37% of nerve terminals still showing obvious signs of pathology. Others have described functional neuromuscular recovery typically beginning after 2wks but rarely complete, 90 days (Makitie and Teravainen 1977b) and 180 days (Fowler *et al.* 1972) after tourniquet induced ischaemia, or 42days following arterial occlusion (Iida *et al.* 2003). In these studies it is unknown which mechanisms are responsible for the recovery processes (*de novo* or collateral innervation) but in YFP/mSOD1 mice, α -motor nerve terminals have been shown to project numerous sprouts 10days after tourniquet induced ischaemia-reperfusion injury (David *et al.* 2007) however it is unknown if the same mechanism would also occur in wild type animals.

6.2 Does the vulnerability of α -motor nerve terminals to hypoxia-reperfusion injury have a wider clinical significance?

The main focus of this study has been the acute hypoxia/ischaemia-injuries caused by tourniquets but perhaps due to the lack of auto-regulated blood supply in the periphery (Smith *et al.* 1977; Low and Tuck 1984), the PNS may also be vulnerable to hypoxic/ischaemic injuries and related pathological event caused by other stimuli.

Cardiovascular, pulmonary, haematological and metabolic disorders

Numerous cardiovascular and pulmonary conditions that cause acute or chronic, systemic or localised hypoxia/ischaemia-reperfusion injuries are now increasingly recognised to trigger peripheral sensorimotor neuropathies that affect significant numbers of patients and have a range of clinical and sub-clinical symptoms (Teunissen *et al.* 2000). These conditions include; atherosclerotic peripheral vascular disease (Chopra and Hurwitz 1969; Nukada *et al.* 1996; McKenzie *et al.* 1999), acute peripheral arterial occlusion (i.e. embolisms: Thomas 1909; Lachance and Daube 1991; Nukada *et al.* 1996), lung cancer (Trojaborg *et al.* 1969), serious lung infections (Caughey *et al.* 1958), chronic obstructive pulmonary disease (Malik *et al.* 1990; Nowak *et al.* 1990), vasculitis (Dyck *et al.* 1987; Fujimura *et al.* 1991; Burns *et al.* 2007b), acute respiratory failure (Gorson and Ropper 1993), polycythemia vera (a condition that results in insufficient numbers of red blood cells: Yiannikas *et al.* 1983), cryoglobulinemia (a condition that results in precipitation of immunoglobulins at low temperatures) and related plasma protein disorders (Gemignani *et al.* 2005; Perez *et al.* 2008; Silberman and Lonial 2008). In many cases, the symptoms of the neuropathic sequelae are more punitive than the primary pathological condition, a situation that is seen in patients suffering peripheral neuropathies because of diabetes mellitus. Diabetes, a condition that affects the ability of insulin to exert control over blood glucose levels, is predicted to affect one in four people by 2025 (Patlak 2002). In itself, diabetes is not a fatal condition as there are numerous therapeutic strategies to help manage blood glucose levels but the neuropathic sequelae of diabetes significantly contribute to its punitive consequences and mortality rate (Vinik *et al.* 2000; Patlak 2002; Dobretsov *et al.* 2007). Between 50% and 90% of diabetic patients suffer from complex and highly variable diabetic peripheral neuropathy, making it the most common form of neuropathy in developed countries (Vinik 1999; Vinik *et al.* 2000; Dobretsov *et al.* 2007). The neuropathy has a distal to proximal progression (which can later affect the autonomic nervous system, e.g., nerve supply to the heart) and triggers positive and negative changes in sensory perception and motor control which often leads to chronic pain, insensitivity to trauma, permanent disability and amputation of limbs. Already described as a dying back neuropathy principally affecting the most distal extremities, morphological changes to neurons including demyelination, axonal atrophy, degeneration and overall loss (Dyck and Giannini 1996; Vinik 1999; Dobretsov *et al.* 2007) but prior to these changes, degeneration and withdrawal of α -motor nerve terminals has been observed (Fahim *et al.* 2000; Ramji *et al.* 2007). The underlying mechanisms of diabetic neuropathy are still not understood however there is considerable evidence to

suggest that metabolic changes and damaged mitochondria within neurons (that in particular, cause ROS formation), and hypoxia/ischaemia due to vascular changes play key roles in its development (Newrick *et al.* 1986; Malik *et al.* 1989; Tesfaye *et al.* 1993; Tesfaye *et al.* 1994; Dyck and Giannini 1996; Vincent *et al.* 2004; Pop-Busui *et al.* 2006; Figueroa-Romero *et al.* 2008; Tannock and King 2008). Based on the results presented here demonstrating the vulnerability of α -motor terminals to hypoxia-reperfusion injury, similar pathological stimuli occurring in diabetes, haematological disorders, cardiovascular and pulmonary diseases may also be causing α -motor nerve terminal pathology and be contributing or possibly causing the neuropathic sequelae associated with these conditions. It is interesting to note that while many of these neuropathic sequelae have a significant sensory component, the preliminary results presented here suggested that the nerve terminals of the muscle spindles that are responsible for proprioception could be less affected by 2H-2R than α -motor nerve terminals. This further highlights the need to build on the preliminary results presented here and further investigate if the nerve terminals of the muscle spindle are vulnerable/resistant to hypoxia-reperfusion injury but also their vulnerability/resistance to other hypoxic/ischaemic injuries and to determine their role in related pathological conditions.

Ageing

Hypoxia may also underlie changes to α -motor nerve terminals and the progressive loss of α -motor neurons as part of the normal ageing process. In healthy humans there is progressive decrease in the partial pressure of arterial O₂ (PaO₂) corresponding to -0.245mmHg (~0.03%) per year, where at 10 years PaO₂ is 85mmHg (~11%), at 50 years PaO₂ 75mmHg (~9.8%) and by 90 years of age PaO₂ is 65mmHg (~8.5%; Crapo *et al.* 1999). Increasing age is also associated with reduced nerve blood flow and decreases in bioenergetic compounds in peripheral nerves (Low *et al.* 1986; Kihara *et al.* 1991) while in skeletal muscle there is loss of capillaries (Degens *et al.* 1993), impairment of contraction-induced vasodilatation (Carlson *et al.* 2008) and decreased blood flow (Tuma *et al.* 1985). Progressive age-related weakening of motor abilities typically begins in the lower extremities and at α -motor nerve terminals, there is an increase in sprouting and synaptic guttering events that eventually leads to denervation and loss of α -motor neurons (Wernig and Herrera 1986; Larsson and Ansved 1995; Verdu *et al.* 2000; Vandervoort 2002; Kernell 2006). Again based on the evidence presented in the current study, it seems possible that systemic and localised decreases in O₂ levels observed in the elderly may be contributing to the normal ageing process of α -motor nerve terminal pathology and loss.

Neurodegenerative disease

Several neurodegenerative diseases, already identified as having nerve terminal loss as a principal event, also have direct links to pathological changes in aerobic respiration, mitochondria or 'oxidative stress'. ALS or Lou Gehrig's disease is the most common adult neurodegenerative disease affecting motor neurons with a lifetime risk of one in 2000, and is characterised by progressive loss of motor

neurons (Bruijn *et al.* 2004). About 20% of familial cases are caused by mutations in SOD1, a vital ROS scavenging enzyme that has been shown to trigger impairment of oxidative phosphorylation, ATP depletion (Mattiazzi *et al.* 2002) and a wave of mitochondrial lysis (Higgins *et al.* 2003). These occur prior to the onset of clinical symptoms, which include early loss of α -motor nerve terminals (Sasaki and Iwata 1996; 1999; Frey *et al.* 2000; Fischer *et al.* 2004; Schaefer *et al.* 2005; Zang da *et al.* 2005). Hereditary Spastic paraplegia, affecting up to one in 10,000 people, is a heterogeneous condition that causes progressive weakness and spasticity in the lower limbs (Filla *et al.* 1992). In an autosomal dominant form, the disease is linked to mutations in Hsp60, a mitochondrial chaperon protein (Hansen *et al.* 2002). In an autosomal recessive form the condition has been linked to loss of the mitochondrial protein, paraplegin, that causes deficits in oxidative phosphorylation (Casari *et al.* 1998) compromised complex 1 activity (see fig 1.1: Atorino *et al.* 2003), and has been shown to cause early morphological changes to synaptic mitochondria prior to degeneration (Ferreirinha *et al.* 2004). Charcot-Marie-Tooth disease sub-form 2A has been linked to defects in mitofusion 2 protein that is involved in mitochondrial fusion (Zuchner *et al.* 2004; Kijima *et al.* 2005; Chung *et al.* 2006). The molecular/cellular events in these diseases are not unlike those caused by hypoxia/ischaemia-reperfusion injury (e.g., mitochondrial impairment, ATP depletion and ROS formation) described here and therefore this study provides a direct link demonstrating that hypoxic/oxidative stress stimuli can cause rapid and selective α -motor nerve pathology.

Contraindications for the use of tourniquets

From the discussion above it would not only suggest that α -motor nerve terminal pathology caused by hypoxic stimuli may be involved in a range of clinical conditions, but also that patients suffering from these various neurodegenerative, cardiovascular, pulmonary, metabolic and haematological conditions may be at an increased risk of α -motor nerve terminal pathology and neurological complications following tourniquet induced ischaemia-reperfusion injuries. This has been shown in the α -motor nerve terminals of mice carrying mSOD1 that have been shown to be significantly more vulnerable compared to their wild-type counterparts (David *et al.* 2007). It has already been noted that children and young adults tend to be more resistant to tourniquet induced ischaemia-reperfusion injuries (Bruner 1951) and elderly are at increased risk because of pre-existing conditions (Kam 2005). Further to this, the elderly may be particularly vulnerable to long-term neurological complications due to tourniquet induced ischaemia-reperfusion injuries. This is not only because of systemic and localised reductions in O₂ levels discussed above, but also because of a reduced ability of α -motor neurons to produce collateral and *de novo* innervations (Verdu *et al.* 2000; Vandervoort 2002; Beal 2005; Apel *et al.* 2009)

6.3 What could be learned from future studies?

From the results presented in the current study and from the discussion above, increasing our understanding of the vulnerability of α -motor nerve terminals to hypoxia-reperfusion injury and related pathological stimuli is of significant clinical importance. In combination with the *ex vivo* model system described here and *in vivo* models that utilise vascular occlusion to trigger ischaemia (thus avoiding potential complications of tourniquet induced mechanical trauma) will provide a powerful means for future studies to address the short and long term pathological consequences, including recovery processes, that occur following hypoxic/ischaemic-injuries. The *ex vivo* model system provides the means to take a reductionist approach to studying hypoxia/ischaemia-reperfusion injuries where many of the individual variables linked with this highly complex pathological stimulus can be monitored and/or controlled. This will help with the identification of key pathological events, important time frames and tolerances for the onset of pathology and cellular/molecular mechanisms of pathogenesis. These studies should be undertaken alongside longitudinal *in vivo* investigations that can examine how individual pathological factors co-evolve to cause ischaemia-reperfusion injuries. Full characterisation of hypoxia/ischemia-reperfusion injuries at α -motor nerve terminals (and other nerve terminals of the PNS) will provide key information on how the long-term neurological complications develop following the use of tourniquet. This type of information can then be used to help establish preventative and therapeutic strategies against tourniquet-induced pathology and other neuropathies where similar pathological stimuli may be occurring. In addition, the model system developed here may also be used to help further our understanding of the mechanisms of α -motor nerve terminal disassembly/loss and the factors that govern selective vulnerability/resistance between different groups of neurons that are increasingly common/recurring themes in the pathogenesis of many neurodegenerative conditions (see Chapter 3). The large size and accessibility of α -motor nerve terminals (and the NMJ/skeletal muscle as a whole) means that a range of systematic structural, functional, pharmacological and genetic studies may be easily carried out in future investigations. Furthermore, the pathological events at α -motor nerve terminals in response to hypoxia-reperfusion injury described here show some important similarities with those known to occur following CNS hypoxic/ischaemic injuries. Continuing studies at α -motor nerve terminals may also form an important paradigm of CNS hypoxic/ischaemic injuries and help in the identification of novel therapeutic targets in both the PNS and CNS.

While this study has focused on the vulnerability of the PNS and in particular α -motor nerve terminals, if looked at from a different and perhaps more classical point of view, α -motor nerve terminals appear remarkably resistant to hypoxic/ischaemic injury. The lack of auto-regulated blood flow (Smith *et al.* 1977; Low and Tuck 1984), intermittent blood flow in resting muscle, rapid rest-to work transitions where skeletal muscle increases its demand for ATP >100times per unit of time (Kernell 2006), lateral forces occluding blood vessels during muscle contractions and reduced blood flow to the limbs in cold environments to maintain core temperatures (Smith *et al.* 1977; Low and

Tuck 1984; Shibata *et al.* 2005; Kernell 2006; Clanton 2007), would suggest that α -motor nerve terminals may be rendered hypoxic/ischaemic on a regular basis. How do α -motor nerve terminals continue to function under these conditions? A considerable amount of research has gone into examining skeletal muscle physiology at rest, in work and in various (potentially) hypoxic environments to understand the complex means of how skeletal muscle buffers against hypoxic insults. Similar studies in α -motor neurons and their nerve terminals have yet to be carried out despite the equal requirement of both for the proficient command and control of skeletal muscle. From the limited studies that have been carried out and in comparison with the CNS, the PNS does appear to have a greater metabolic safety factor with ~10% of the oxygen requirement but similar ATP levels, and better anaerobic abilities (Stewart *et al.* 1965; Campa and Engel 1971; Low *et al.* 1985; Zollman *et al.* 1991; Mohler *et al.* 1999). Are these adaptations alone sufficient to buffer α -motor neurons and their nerve terminals against transient hypoxic insults? When compared to the complex adaptations of skeletal muscle, it seems likely that we may have more to learn about how α -motor neurons and their nerve terminals function under hypoxic conditions. In the current study, I described preliminary work that made the first steps to address this issue by trying to determine if the novel oxygen storing protein, neuroglobin (NGB), was expressed in α -motor nerve terminals (Chapter 5). While this needs to be the subject to further study and the results are currently only preliminary, the results suggested that NGB could be expressed at the mouse NMJ and if it is expressed, may indicate a role in buffering against hypoxia insults. If NGB is expressed at the NMJ, what other factors at the NMJ as a whole help maintain ATP levels during hypoxic insults? Addressing this issue would not only improve our understanding of the physiology of α -motor neurons, their nerve terminals and the NMJ in general, but may also provide targets to direct novel therapeutic strategies against hypoxia/ischaemic-reperfusion injuries and other conditions with related pathological stimuli in the future.

6.4 Summary

Nerve terminals are known to be vulnerable to a range of toxic, traumatic and neurodegenerative disease stimuli, but it was unknown if the nerve terminals of α -motor neurons were vulnerable to hypoxic injury. In response to my aim, I have demonstrated that α -motor nerve terminals are highly and selectively vulnerable to hypoxia-reperfusion injury. This finding may not only have direct implications for the use of surgical and non-surgical tourniquets but also in a range of other disease processes where similar hypoxic/ischaemic stimuli maybe causing or contributing to the development of punitive neuropathies.

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Appendix I

Data Tables

Data Tables

In the following appendix is the tabulated data for Chapters 2 and 3 which includes raw data, % data and summary statistics. Tables 1-3 show α -motor nerve terminal response, Tables 4 show observations of muscle spindle innervation.

Table 1: α -motor nerve terminals response from 8-12wk old C57Bl/6 mice

- a) Lumbrical control data
- b) Lumbrical 2H-2R data
- c) TA control data
- d) TA 2H-2R data
- e) TS control data
- f) TS 2H-2R data

Table 2: α -motor nerve terminals response from 5-6wk old C57Bl/6 mice

- a) Lumbrical control data
- b) Lumbrical 2H-2R data
- c) TA control data
- d) TA 2H-2R data
- e) TS control data
- f) TS 2H-2R data

Table 3: α -motor nerve terminals response from 5-6wk old '*Wld^s*' mice

- a) Lumbrical control data
- b) Lumbrical 2H-2R data
- c) TA control data
- d) TA 2H-2R data
- e) TS control data
- f) TS 2H-2R data

Table 4: Observations of IA nerve terminal and γ -NMJ from lumbrical muscles

- a) 8-12wk old C57Bl/6 control data
- b) 8-12wk old C57Bl/6 2H-2R data
- c) 5-6wk old C57Bl/6 control data
- d) 5-6wk old C57Bl/6 2H-2R data
- e) 5-6wk old *Wld^s* control data
- f) 5-6wk old *Wld^s* 2H-2R data

Table 1(a) Control data from lumbrical muscles from 8-12wk old C57Bl/6 mice

N	RAW DATA				% DATA		
	Vacant	Partial	Full	TOTAL	%Vacant	%Partial	%Full
1	4	2	211	217	1.84	0.92	97.24
2	0	0	147	147	0.00	0.00	100.00
3	1	1	113	115	0.87	0.87	98.26
4	4	0	199	203	1.97	0.00	98.03
1	2	0	149	151	1.32	0.00	98.68
2	0	0	182	182	0.00	0.00	100.00
3	0	1	139	140	0.00	0.71	99.29
4	0	1	170	171	0.00	0.58	99.42
1	2	3	273	278	0.72	1.08	98.20
2	1	0	226	227	0.44	0.00	99.56
3	0	0	141	141	0.00	0.00	100.00
4	0	0	164	164	0.00	0.00	100.00
1	2	0	261	263	0.76	0.00	99.24
2	0	0	208	208	0.00	0.00	100.00
3	0	0	151	151	0.00	0.00	100.00
4	6	0	173	179	3.35	0.00	96.65
1	0	0	206	206	0.00	0.00	100.00
2	0	0	219	219	0.00	0.00	100.00
3	0	0	193	193	0.00	0.00	100.00
4	0	0	186	186	0.00	0.00	100.00
1	3	0	216	219	1.37	0.00	98.63
2	2	0	172	174	1.15	0.00	98.85
3	1	0	139	140	0.71	0.00	99.29
4	0	0	167	167	0.00	0.00	100.00
1	0	0	145	145	0.00	0.00	100.00
2	2	0	221	223	0.90	0.00	99.10
3	0	0	103	103	0.00	0.00	100.00
4	1	0	177	178	0.56	0.00	99.44
TOTAL	31	8	5051	5090			

Summary		Statistics		
animals (N)	/		% Vacant	% Partial
muscles (n)	28	%MEAN	0.57	0.15
endplates	5051	%SEM	0.15	0.06
				0.17

NOTES: N = the animal number, where n = the number of lumbrical muscles. Each lumbrical muscle is numbered from 1-4 according to anatomical location from the hallux/big toe. % Data calculated as the population percentage per lumbrical (n) and then pooled. Statistics calculated with pooled data. SEM= standard error of the mean

Table 1(b) 2H-2R data from lumbrical muscles from 8-12wk old C57Bl/6 mice

N	RAW DATA				% DATA		
	Vacant	Partial	Full	TOTAL	%Vacant	%Partial	%Full
1	237	1	2	240	98.75	0.42	0.83
2	198	1	7	206	96.12	0.49	3.40
3	122	1	14	137	89.05	0.73	10.22
4	167	0	37	204	81.86	0.00	18.14
1	189	1	9	199	94.97	0.50	4.52
2	184	0	5	189	97.35	0.00	2.65
3	138	0	0	138	100.00	0.00	0.00
4	129	2	25	156	82.69	1.28	16.03
1	217	0	13	230	94.35	0.00	5.65
2	216	0	1	217	99.54	0.00	0.46
3	179	0	1	180	99.44	0.00	0.56
4	162	0	5	167	97.01	0.00	2.99
1	218	5	26	249	87.55	2.01	10.44
2	202	3	16	221	91.40	1.36	7.24
3	209	4	10	223	93.72	1.79	4.48
4	94	2	49	145	64.83	1.38	33.79
1	117	11	78	206	56.80	5.34	37.86
2	129	11	24	164	78.66	6.71	14.63
3	89	2	30	121	73.55	1.65	24.79
4	47	4	76	127	37.01	3.15	59.84
1	175	3	5	183	95.63	1.64	2.73
2	192	1	14	207	92.75	0.48	6.76
3	99	1	11	111	89.19	0.90	9.91
4	57	3	63	123	46.34	2.44	51.22
1	154	1	4	159	96.86	0.63	2.52
2	141	5	18	164	85.98	3.05	10.98
3	129	0	28	157	82.17	0.00	17.83
4	10	3	138	151	6.62	1.99	91.39
TOTAL	4200	65	709	4974			

Summary		Statistics		
Total animals (N)	/		% Vacant	% Partial
Total muscles (n)	28	%MEAN	82.51	1.35
Total endplates	4974	%SEM	4.16	0.31
				4.02

Table 1(c) Control data from TA muscles from 8-12wk old C57Bl/6 mice

N/n	RAW DATA				% DATA			
	Vacant	Partial	Full	TOTAL	%Vacant	%Partial	%Full	%TOTAL
1			651	651	0.00	0.00	100.00	100
2			759	759	0.00	0.00	100.00	100
3	0	0	713	713	0.00	0.00	100.00	100
4			<i>examined but not quantified</i>					
To	0	0	2123	2123				
Summary				Statistics				
animals (N)				3	% Vacant	% Partial	% Full	
muscles (n)				3	%MEAN	0.00	0.00	100.00
endplates				2123	%SEM	0.00	0.00	0.00

Table 1(d) 2H-2R data from TA muscles from 8-12wk old C57Bl/6 mice

N/n	RAW DATA				% DATA			
	Vacant	Partial	Full	TOTAL	%Vacant	%Partial	%Full	%TOTAL
1	268	28	110	406	66.01	6.90	27.09	100
2	392	24	74	490	80.00	4.90	15.10	100
3	258	18	159	435	59.31	4.14	36.55	100
4	110	14	298	422	26.07	3.32	70.62	100
To	1028	84	641	1753				
Summary				Statistics				
animals (N)				4	% Vacant	% Partial	% Full	
muscles (n)				4	%MEAN	57.85	4.81	37.34
endplates				1753	%SEM	11.44	0.77	11.93

Table 1(e) Control data from TS muscles from 8-12wk old C57Bl/6 mice

N/n	RAW DATA				% DATA			
	Vacant	Partial	Full	TOTAL	%Vacant	%Partial	%Full	%TOTAL
1	2	2	669	673	0.30	0.30	99.41	100
2	0	0	727	727	0.00	0.00	100.00	100
3	0	0	669	669	0.00	0.00	100.00	100
4			<i>examined but not quantified</i>					
To	2	2	2065	2069				
Summary				Statistics				
animals (N)				3	% Vacant	% Partial	% Full	
muscles (n)				3	%MEAN	0.10	0.10	99.80
endplates				2069	%SEM	0.10	0.10	0.20

Table 1(f) 2H-2R data from TS muscles from 8-12wk old C57Bl/6 mice

N/n	RAW DATA				% DATA			
	Vacant	Partial	Full	TOTAL	%Vacant	%Partial	%Full	%TOTAL
1	109	10	254	373	29.22	2.68	68.10	100
2	156	8	317	481	32.43	1.66	65.90	100
3	178	28	207	413	43.10	6.78	50.12	100
4	31	9	458	498	6.22	1.81	91.97	100
To	474	55	1236	1765				
Summary				Statistics				
animals (N)				4	% Vacant	% Partial	% Full	
muscles (n)				4	%MEAN	27.74	3.23	69.02
endplates				1765	%SEM	7.77	1.21	8.64

NOTES: N = the animal number, where n = the number of lumbrical muscles. Each lumbrical muscle is numbered from 1-4 according to anatomical location from the hallux/big toe. % Data calculated as the population percentage per lumbrical (n) and then pooled. Statistics calculated with pooled data. SEM= standard error of the mean, To =total

Table 2(a) Control data from lumbrical muscles from 5-6wk old C57Bl/6 mice

N	n	RAW DATA				% DATA		
		Vacant	Partial	Full	TOTAL	%Vacant	%Partial	%Full
4	1	0	0	225	225	0.00	0.00	100.00
	2	1	0	225	226	0.44	0.00	99.56
	3	8	3	193	204	3.92	1.47	94.61
	4	0	0	280	280	0.00	0.00	100.00
3	1	0	0	180	180	0.00	0.00	100.00
	2	0	0	214	214	0.00	0.00	100.00
	3	0	0	165	165	0.00	0.00	100.00
	4	0	0	217	217	0.00	0.00	100.00
2	1	0	0	284	284	0.00	0.00	100.00
	2	0	0	221	221	0.00	0.00	100.00
	3	24	2	167	193	12.44	1.04	86.53
	4	0	0	222	222	0.00	0.00	100.00
1	1	0	0	225	225	0.00	0.00	100.00
	2	0	1	261	262	0.00	0.38	99.62
	3	0	0	210	210	0.00	0.00	100.00
	4	0	0	165	165	0.00	0.00	100.00
TOTAL		33	6	3454	3493			

Summary		Statistics			
animals (N)	4		% Vacant	% Partial	% Full
muscles (n)	16	%MEAN	1.05	0.18	98.77
endplates	3493	%SEM	0.80	0.11	0.88

Table 2(b) 2H-2R data from lumbrical muscles from 5-6wk old C57Bl/6 mice

N	n	RAW DATA				% DATA		
		Vacant	Partial	Full	TOTAL	%Vacant	%Partial	%Full
4	1	247	2	12	261	94.64	0.77	4.60
	2	199	2	4	205	97.07	0.98	1.95
	3	218	4	4	226	96.46	1.77	1.77
	4	115	3	57	175	65.71	1.71	32.57
3	1	260	2	4	266	97.74	0.75	1.50
	2	235	4	6	245	95.92	1.63	2.45
	3	200	7	13	220	90.91	3.18	5.91
	4	54	4	86	144	37.50	2.78	59.72
2	1	286	4	44	334	85.63	1.20	13.17
	2	289	0	0	289	100.00	0.00	0.00
	3	218	0	38	256	85.16	0.00	14.84
	4	148	20	39	207	71.50	9.66	18.84
1	1	230	4	12	246	93.50	1.63	4.88
	2	247	0	2	249	99.20	0.00	0.80
	3	182	14	20	216	84.26	6.48	9.26
	4	98	3	57	158	62.03	1.90	36.08
TOTAL		3226	73	398	3697			

Summary		Statistics			
Total animals (N)	4		% Vacant	% Partial	% Full
Total muscles (n)	16	%MEAN	84.83	2.15	13.02
Total endplates	3697	%SEM	4.34	0.64	4.16

NOTES: N = the animal number, where n = the number of lumbrical muscles. Each lumbrical muscle is numbered from 1-4 according to anatomical location from the hallux/big toe. % Data calculated as the population percentage per lumbrical (n) and then pooled. Statistics calculated with pooled data. SEM= standard error of the mean

Table 2(c) Control data from TA muscles from 5-6wk old C57Bl/6 mice

N/n	RAW DATA			% DATA		
	Vacant	Partial	Full	%Vacant	%Partial	%Full
1	0	0	360	0.00	0.00	100.00
2	0	0	449	0.00	0.00	100.00
3	0	0	572	0.00	0.00	100.00
4			<i>examined but not quantified</i>			
To	0	0	1381			

Summary			Statistics		
animals (N)		3	% Vacant	% Partial	% Full
muscles (n)		3	%MEAN	0.00	100.00
endplates		1381	%SEM	0.00	0.00

Table 2(d) 2H-2R data from TA muscles from 5-6wk old C57Bl/6 mice

N/n	RAW DATA			% DATA		
	Vacant	Partial	Full	%Vacant	%Partial	%Full
1	638	1	0	99.84	0.16	0.00
2	485	3	3	98.78	0.61	0.61
3	539	2	0	99.63	0.37	0.00
4	431	2	0	99.54	0.46	0.00
To	2093	8	3			

Summary			Statistics		
animals (N)		4	% Vacant	% Partial	% Full
muscles (n)		4	%MEAN	99.45	0.40
endplates		2104	%SEM	0.23	0.15

Table 2(e) Control data from TS muscles from 5-6wk old C57Bl/6 mice

N/n	RAW DATA			% DATA		
	Vacant	Partial	Full	%Vacant	%Partial	%Full
1	0	0	567	0.00	0.00	100.00
2	5	0	660	0.75	0.00	99.25
3	0	0	680	0.00	0.00	100.00
4			<i>examined but not quantified</i>			
To	5	0	1907			

Summary			Statistics		
animals (N)		3	% Vacant	% Partial	% Full
muscles (n)		3	%MEAN	0.25	0.00
endplates		1921	%SEM	0.25	0.00

Table 2(f) 2H-2R data from TS muscles from 5-6wk old C57Bl/6 mice

N/n	RAW DATA			% DATA		
	Vacant	Partial	Full	%Vacant	%Partial	%Full
1	538	14	13	95.22	2.48	2.30
2	535	14	17	94.52	2.47	3.00
3	583	1	0	99.83	0.17	0.00
4	503	1	1	99.60	0.20	0.20
To	2159	30	31			

Summary			Statistics		
animals (N)		4	% Vacant	% Partial	% Full
muscles (n)		4	%MEAN	97.29	1.33
endplates		2220	%SEM	1.41	0.66

NOTES: N = the animal number, where n = the number of lumbrical muscles. Each lumbrical muscle is numbered from 1-4 according to anatomical location from the hallux/big toe. % Data calculated as the population percentage per lumbrical (n) and then pooled. Statistics calculated with pooled data. SEM= standard error of the mean, To =total

Table 3(a) Control data from lumbrical muscles from 5-6wk old 'Wld's' mice

N	RAW DATA				% DATA		
	Vacant	Partial	Full	TOTAL	%Vacant	%Partial	%TOTAL
1				0			0
2				0			0
3				0			0
4				0			0
1				0			0
2							
3							
4							
1							
2							
3							
4							
1	7	1	242	250	2.80	0.40	96.80
2	1	2	308	311	0.32	0.64	99.04
3	5	0	220	225	2.22	0.00	97.78
4	0	0	212	212	0.00	0.00	100.00
1	1	3	254	258	0.39	1.16	98.45
2	0	2	258	260	0.00	0.77	99.23
3	2	0	211	213	0.94	0.00	99.06
4	5	1	129	135	3.70	0.74	95.56
1	0	2	264	266	0.00	0.75	99.25
2	1	1	225	227	0.44	0.44	99.12
3	0	0	179	179	0.00	0.00	100.00
4	2	2	176	182	1.10	1.10	97.80
TOTAL	24	14	2680	2718			

Summary		Statistics		
animals (N)	3		% Vacant	% Partial
muscles (n)	12	%MEAN	0.99	0.50
endplates	2718	%SEM	0.36	0.12
				0.38

NOTES: N = the animal number, where n = the number of lumbrical muscles. Each lumbrical muscle is numbered from 1-4 according to anatomical location from the hallux/big toe. % Data calculated as the population percentage per lumbrical (n) and then pooled. Statistics calculated with pooled data. SEM= standard error of the mean

Table 3(b) 2H-2R data from lumbrical muscles from 5-6wk old 'wld's' mice

N	RAW DATA				% DATA		
	Vacant	Partial	Full	TOTAL	%Vacant	%Partial	%TOTAL
1	274	4	17	295	92.88	1.36	5.76
2	200	0	60	260	76.92	0.00	23.08
3	133	0	4	137	97.08	0.00	2.92
4	89	4	27	120	74.17	3.33	22.50
1	201	5	29	235	85.53	2.13	12.34
2	215	0	5	220	97.73	0.00	2.27
3	205	1	1	207	99.03	0.48	0.48
4	43	3	3	49	87.76	6.12	6.12
1	192	12	79	283	67.84	4.24	27.92
2	312	2	6	320	97.50	0.63	1.88
3	186	1	23	210	88.57	0.48	10.95
4	101	5	59	165	61.21	3.03	35.76
1	211	8	99	318	66.35	2.52	31.13
2	260	3	2	265	98.11	1.13	0.75
3	193	2	34	229	84.28	0.87	14.85
4	145	6	49	200	72.50	3.00	24.50
1	186	4	67	257	72.37	1.56	26.07
2	278	0	16	294	94.56	0.00	5.44
3	187	3	13	203	92.12	1.48	6.40
4	115	3	112	230	50.00	1.30	48.70
1	177	2	108	287	61.67	0.70	37.63
2	285	0	7	292	97.60	0.00	2.40
3	153	0	28	181	84.53	0.00	15.47
4	163	1	47	211	77.25	0.47	22.27
TOTAL	4504	69	895	5468			

Summary		Statistics		
Total animals (N)	6		% Vacant	% Partial
Total muscles (n)	24	%MEAN	82.40	1.45
Total endplates	5468	%SEM	2.88	0.32
				2.78

Table 3(c) control data from TA muscles from 5-6wk old 'Wlds' mice

N/n	RAW DATA			% DATA		
	Vacant	Partial	Full	%Vacant	%Partial	%Full
1	0	0	637	0.00	0.00	100.00
2	0	0	676	0.00	0.00	100.00
3	0	0	621	0.00	0.00	100.00
4						
5						
To	0	0	1934			

Summary			Statistics		
animals (N)		3	% Vacant	% Partial	% Full
muscles (n)		3	%MEAN	0.00	100.00
endplates		1934	%SEM	0.00	0.00

Table 3(e) Control data from TS muscles from 5-6wk old 'Wlds' mice

N/n	RAW DATA			% DATA		
	Vacant	Partial	Full	%Vacant	%Partial	%Full
1	0	0	668	0.00	0.00	100.00
2	0	0	563	0.00	0.00	100.00
3	0	0	663	0.00	0.00	100.00
4						
5						
To	0	0	1894			

Summary			Statistics		
animals (N)		3	% Vacant	% Partial	% Full
muscles (n)		3	%MEAN	0.00	100.00
endplates		1894	%SEM	0.00	0.00

NOTES: N = the animal number, where n = the number of lumbrical muscles. Each lumbrical muscle is numbered from 1-4 according to anatomical location from the hallux/big toe. % Data calculated as the population percentage per lumbrical (n) and then pooled. Statistics calculated with pooled data. SEM= standard error of the mean, To =total

Table 3(d) 2H-2R data from TA muscles from 5-6wk old 'Wlds' mice

N/n	RAW DATA			% DATA		
	Vacant	Partial	Full	%Vacant	%Partial	%Full
1	510	10	14	95.51	1.87	2.62
2	647	3	0	99.54	0.46	0.00
3	479	7	3	97.96	1.43	0.61
4	434	6	1	98.41	1.36	0.23
5	450	4	0	99.12	0.88	0.00
To	2520	30	18			

Summary			Statistics		
animals (N)		5	% Vacant	% Partial	% Full
muscles (n)		5	%MEAN	98.11	1.20
endplates		2568	%SEM	0.71	0.24

Table 3(f) 2H-2R data from TS muscles from 5-6wk old 'Wlds' mice

N/n	RAW DATA			% DATA		
	Vacant	Partial	Full	%Vacant	%Partial	%Full
1	449	20	15	92.77	4.13	3.10
2	528	8	14	96.00	1.45	2.55
3	471	1	0	99.79	0.21	0.00
4	373	5	4	97.64	1.31	1.05
5	390	7	5	97.01	1.74	1.24
To	2211	41	38			

Summary			Statistics		
animals (N)			% Vacant	% Partial	% Full
muscles (n)			%MEAN	96.64	1.77
endplates			%SEM	1.15	0.65

Table 4(a) Observed presence of muscle spindle innervations in control lumbrical muscle from 8-12wks C57Bl/6 mice

≥	≤	Control	
		IA/II ending	γ-NMJ
7	1		
	2	excluded	
	3		
	4		
6	1	yes	yes
	2	no	yes
	3	no	yes
	4	no	yes
5	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	no	yes
4	1	yes	yes
	2	no	yes
	3	yes	yes
	4	no	yes
3	1	yes	yes
	2	no	yes
	3	yes	yes
	4	no	yes
2	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	yes
1	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	yes

Summary		
animals (<i>N</i>)		6
muscles (<i>n</i>)		24
Statistics		
	IA/II ending	γ-NMJ
Total 'yes'	16	24
% mean 'yes'	66.67	100

Table 4(b) Observed presence of muscle spindle innervations in 2H-2R lumbrical muscle from 8-12wks C57Bl/6 mice

≥	≤	2H-2R	
		IA/II ending	γ-NMJ
7	1	yes	yes
	2	yes	yes
	3	no	yes
	4	no	yes
6	1	yes	yes
	2	yes	no
	3	yes	yes
	4	no	no
5	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	no	yes
4	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	no	yes
3	1	no	no
	2	yes	yes
	3	yes	yes
	4	no	yes
2	1	yes	yes
	2	yes	yes
	3	no	no
	4	yes	yes
1	1	yes	yes
	2	yes	yes
	3	no	yes
	4	no	yes

Summary		
animals (<i>N</i>)		7
muscles (<i>n</i>)		28
Statistics		
	IA/II ending	γ -NMJ
Total 'yes'	18	24
% mean 'yes'	75	85.71

NOTES: The total number of muscles observed with at least 1 morphologically normal IA/II nerve terminal endings and/or g-NMJ in the 'Total yes' rows. '% mean yes' values calculated by ('total yes'/muscle (n))x100).

Table 4(c) Observed presence of muscle spindle innervations in control lumbrical muscle from 5-6wks C57Bl/6 mice

Σ	n	Control	
		IA/II ending	γ-NMJ
4	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	yes
3	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	yes
2	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	yes
1	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	yes

Summary		
animals (<i>N</i>)		4
muscles (<i>n</i>)		16
Statistics		
	IA/II ending	γ-NMJ
Total 'yes'	16	16
% Total 'yes'	100	100

Table 4(d) Observed presence of muscle spindle innervations in 2H-2R lumbrical muscle from 5-6wks C57Bl/6 mice

Σ	n	2H-2R	
		IA/II ending	γ-NMJ
4	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	no	yes
3	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	no	no
2	1	no	yes
	2	no	yes
	3	yes	yes
	4	no	yes
1	1	yes	yes
	2	yes	yes
	3	no	yes
	4	no	no

Summary		
animals (N)		4
muscles (n)		16
Statistics		
	IA/II ending	γ -NMJ
Total 'yes'	9	14
% Total 'yes'	56.25	87.5

NOTES: The total number of muscles observed with at least 1 morphologically normal IA/II nerve terminal endings and/or g-NMJ in the 'Total yes' rows. '% mean yes' values calculated by ('total yes'/muscle (n'))x100).

Table 4(e) Observed presence of muscle spindle innervations in control lumbrical muscle from 5-6wks Wlds mice

Σ	n	Control	
		IA/II ending	γ-NMJ
7	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	no
6	1	yes	yes
	2	yes	yes
	3	no	yes
	4	no	no
5	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	no	yes
4	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	yes
3	1	yes	yes
	2	yes	yes
	3	no	yes
	4	no	no
2	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	no	yes
1	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	yes

Summary		
animals (N)		7
muscles (n)		28
Statistics		
	IA/II ending	γ-NMJ
Total 'yes'	22	25
% Total 'yes'	78.6	89.3

Table 4(f) Observed presence of muscle spindle innervations in 2H-2R lumbrical muscle from 5-6wks Wlds mice

Σ	n	2H-2R	
		IA/II ending	γ-NMJ
7	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	no
6	1	yes	yes
	2	yes	yes
	3	no	yes
	4	no	yes
5	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	no	yes
4	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	no	yes
3	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	yes
2	1	yes	yes
	2	yes	yes
	3	no	yes
	4	no	no
1	1	yes	yes
	2	yes	yes
	3	yes	yes
	4	yes	yes

Summary		
animals (N)		7
muscles (n)		28
Statistics		
	IA/II ending	γ-NMJ
Total 'yes'	22	26
% Total 'yes'	78.6	92.9

NOTES: The total number of muscles observed with at least 1 morphologically normal IA/II nerve terminal endings and/or g-NMJ in the 'Total yes' rows. '% mean yes' values calculated by ('total yes'/muscle (n))x100).

Appendix II

Publications

Research Publications (abstracts only)

Full copies of the following papers can be found as PDF files on attached DVD.

Baxter B, Gillingwater TH, Parson SH (2008) "Rapid loss of motor nerve terminals following hypoxia-reperfusion injury occurs via mechanisms distinct from classic Wallerian degeneration." *Journal of Anatomy* 212(6):827-835

Motor nerve terminals are known to be vulnerable to a wide range of pathological stimuli. To further characterize this vulnerability, we have developed a novel model system to examine the response of mouse motor nerve terminals in ex vivo nerve/muscle preparations to 2 h hypoxia followed by 2 h reperfusion. This insult induced a rapid loss of neurofilament and synaptic vesicle protein immunoreactivity at pre-synaptic motor nerve terminals but did not appear to affect post-synaptic endplates or muscle fibres. The severity of nerve terminal loss was dependent on the age of the mouse and muscle type: in 8-12-week-old mice the predominantly fast-twitch lumbrical muscles showed an 82.5% loss, whereas the predominantly slow-twitch muscles transversus abdominis and triangularis sterni showed a 57.8% and 27.2% loss, respectively. This was contrasted with a > 97% loss in the predominantly slow-twitch muscles from 5-6-week-old mice. We have also demonstrated that nerve terminal loss occurs by a mechanism distinct from Wallerian degeneration, as the slow Wallerian degeneration (Wld(s)) gene did not modify the extent of nerve terminal pathology. Together, these data show that our new model of hypoxia-reperfusion injury is robust and repeatable, that it induces rapid, quantitative changes in motor nerve terminals and that it can be used to further examine the mechanisms regulating nerve terminal vulnerability in response to hypoxia-reperfusion injury.

Bettini NL, Moores TS, Baxter B, Deuchars J, Parson SH (2007) "Dynamic remodelling of synapses can occur in the absence of the parent cell body." *BMC Neurosci* Sep 26; 8:79

BACKGROUND: Retraction of nerve terminals is a characteristic feature of development, injury and insult and may herald many neurodegenerative diseases. Although morphological events have been well characterized, we know relatively little about the nature of the underlying cellular machinery. Evidence suggests a strong local component in determining which neuronal branches and synapses are lost, but a greater understanding of this basic neurological process is required. Here we test the hypothesis that nerve terminals are semi-autonomous and able to rapidly respond to local stimuli in the absence of communication with their parent cell body. **RESULTS:** We used an isolated preparation consisting of distal peripheral nerve stumps, associated nerve terminals and post-synaptic muscle fibres, maintained in-vitro for up to 3 hrs. In this system synapses are intact but the presynaptic nerve terminal is disconnected from its cell soma. In control preparations synapses were stable for extended periods and did not undergo Wallerian degeneration. In contrast, addition of purines triggers rapid changes at synapses. Using fluorescence and electron microscopy we observe ultrastructural and gross morphological events consistent with nerve terminal retraction. We find no evidence of Wallerian or Wallerian-like degeneration in these preparations. Pharmacological experiments implicate pre-synaptic P2X7 receptor subunits as key mediators of these events. **CONCLUSION:** The data presented suggest; first that isolated nerve terminals are able to regulate connectivity independent of signals from the cell body, second that synapses exist in a dynamic state, poised to shift from stability to loss by activating intrinsic mechanisms and molecules, and third that local purines acting at purinergic receptors can trigger these events. A role for ATP receptors in this is not surprising since they are frequently activated during cellular injury, when adenosine triphosphate is released from damaged cells. Local control demands that the elements necessary to drive retraction are constitutively present. We hypothesize that pre-existing scaffolds of

molecular motors and cytoskeletal proteins could provide the dynamism required to drive such structural changes in nerve terminals in the absence of the cell body.

Baxter RL, Deuchars J, Parson SH (2005) “A₂A Adenosine Receptors are Located on Presynaptic Motor Nerve Terminals in the Mouse”. *Synapse* 57:229-234

Extracellular adenosine is present at the mammalian neuromuscular junction (NMJ) by virtue of its release from activated nerve terminals and muscle fibres, and as a metabolite of adenosine tri-phosphate, which is coreleased with acetylcholine. Two activities for adenosine have been described: an inhibitory effect presumed to be modulated by the A₁ receptor subtype, and a facilitatory effect mediated by the A₂A receptor subtype. To date, only pharmacological evidence is available for these actions. We have used an antibody against the A₂A receptor subtype, and demonstrated that A₂A receptors are present on presynaptic motor nerve terminals at NMJs but not on associated glial or muscle cells, in the mouse. These results therefore provide additional evidence that there are multiple adenosine receptors present at the NMJ, and that stimulation of quantal and nonquantal release of acetylcholine (ACh) could be mediated by A₂A receptors.

Poster Abstracts

B. Baxter, M.J. Parsons, T. H. Gillingwater & S. H. Parson (2009) "Hypoxia induces rapid and selective injury to α -motor nerve terminals." *Journal of Anatomy*.(awaiting publication) PX Intra-uterine environment and Placentation , University of Nottingham 2-4 July 2008, Anatomical Society of Great Britain & Ireland

A growing body of evidence shows that presynaptic nerve terminals throughout the nervous system are vulnerable to a range of traumatic, toxic and disease-related neurodegenerative stimuli. Using a novel *ex vivo* model, we have recently shown that α -motor nerve terminals in several mouse muscles are highly susceptible to hypoxia-reperfusion injury. 2hr hypoxia ($<0.25\% O_2$) followed by 2hr reperfusion in lumbrical muscles triggered loss of neurofilament 168kDa (NF) and synaptic vesicle 2 (SV2) protein immunoreactivity in 82.5% ($n=28$) of presynaptic α -motor nerve terminals. This insult did not appear to affect post-synaptic endplates or muscle fibres. We also established that this loss of α -motor nerve terminal morphology occurs by a mechanism distinct from Wallerian degeneration, as the *slow Wallerian degeneration* (*Wld^s*) gene did not protect nerve terminals from these pathological changes. We now show that 1A muscle spindle afferents and γ -motor terminals appear to be more resistant to hypoxia-reperfusion injury compared with α -motor nerve terminals. Furthermore, we now demonstrate that significant loss of α -motor nerve terminals in mouse lumbrical muscles occurs in response to hypoxia alone, without reperfusion. A time series analysis shows that loss of NF/ SV2 immunoreactivity in α -motor nerve terminals first appears after a 1.5hr hypoxia insult and significant loss appears after 2hr, 23% ($n=20$) and 44.1% ($n=20$) loss respectively. Collectively, this data suggests that α -motor nerve terminals are highly and selectively vulnerable to hypoxic injury and that pathology is rapidly induced within 1.5hr of hypoxia alone. These findings may have clinical implications for the use of surgical tourniquets and in the etiology of many neurodegenerative diseases where mechanisms relating to hypoxia and hypoxia-reperfusion injury have been implicated.

Baxter, B.; Gillingwater, T. H.; Parson, S. H (2008). "Vulnerability of motor nerve terminals to hypoxia." *Journal of Anatomy*. 212(1):81. P2 Advances in Stem Cell Biology, University of Durham 3-5 July 2007 Anatomical Society of Great Britain & Ireland

Damage to neurons caused by ischemia and/or ischaemia-reperfusion injuries are now thought to contribute to the neurological defects seen as a consequence of surgery, cardio-pulmonary failure and stroke. There is growing evidence to indicate that compared with the axon and cell body, the nerve terminal/synaptic compartment of neurons is especially vulnerable to functional and morphological changes in response to pathological stimuli. To investigate if nerve terminals are vulnerable to ischaemic-reperfusion injury we have developed an *ex-vivo* model utilizing the mouse neuromuscular junction. Preliminary work has been based on the hypoxic element of ischaemia as this is largely believed to be the most damaging. This work suggests that 2 hours of hypoxia can selectively trigger rapid disruption and apparent loss of motor nerve terminal morphology. The heterogeneity of the morphological responses to hypoxia indicates that a) several mechanisms of loss may be active and b) these mechanisms may, at least in part, be controlled locally. It is likely that these morphological changes would have an adverse effect on synaptic function and connectivity. This therefore, may contribute to some of the punitive symptoms following ischaemia/ischaemia-reperfusion injuries in the peripheral nervous system.

Baxter, R.; Parson, S. H (2007) “Does hypoxia prime mouse motor nerve terminal loss?” *Journal of Anatomy*. 210(5):626-627.P14 Neuroanatomy: Centenary of Cajal, University of Madrid, 13-15th September 2005, Joint Meeting of the Anatomical Society of Great Britain and Ireland and the Spanish Anatomical Society

Changes to or loss of synaptic connectivity as a result of neuronal death causes either aberrant or complete loss of function of post-synaptic targets. It is this loss of synaptic connectivity that causes the punitive symptoms seen in neuropathological disorders. Recently, changes to and loss of nerve terminals has been shown to occur before cell body death and prior to the manifestation of clinical symptoms in a number of neuropathies, e.g., Alzheimer's disease. Understanding the mechanisms and triggers of nerve terminal loss are therefore of key importance. Using the neuromuscular junction as a model synapse, mouse skeletal muscle explants with long distal nerve stumps were maintained for >3 hours. We have shown that nerve terminals appear to be quickly lost in response to adenosine triphosphate (ATP) and hypoxia. In wild type mice, this appears as a significant reduction to neurofilament and synaptic vesicle immunoreactivity, indicative of gross morphological change in >3 hours. Real time monitoring of nerve terminals using transgenic Thy-1/16(YFP)/C57Bl mice that express yellow fluorescent protein in all their neurons reveal that degenerative changes can occur over minute timescales. This work suggests that nerve terminals may be very quickly lost during ischemia where increases in extracellular ATP are coupled with reduced oxygen availability. Using YFP transgenic mice we will investigate previous unpublished findings that 3'-O-(4-benzoyl)benzoyl adenosine triphosphate (BzATP) causes adult motor nerve terminal withdrawal. Future work will include investigations into the morphological and cellular events induced by ischaemic insult, and in particular, the involvement of the cytoskeletal as a possible mediator of nerve terminal stability, degeneration and withdrawal.